INTERACTION OF AMATOXINS WITH PLANT CELLS AND RNA POLYMERASES II: SELECTION OF AMANITIN-RESISTANT CELL LINES AND SYNTHESIS OF AMANITIN-BASED AFFINITY LIGANDS

By

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Major Department: Microbiology and Cell Science

A series of experiments directed toward deriving basic information regarding plant RNA polymerase II is presented. The experiments described herein relate to the potential of isolating RNA polymerase II mutants in plants, using carrot cell cultures as models. Additionally, the synthesis of amanitin-based affinity ligands to immobilize isolated plant RNA polymerase II and associated transcriptional complexes is described.

RNA polymerase II activities have been isolated from suspension cultures of carrot and compared to other plant RNA polymerases II with respect to subunit analysis and inhibition with α-amanitin. RNA polymerase II purified by polymin P adsorption, DE52, phosphocellulose, and DNA-agarose chromatography is shown to copurify with proteins of

175 (and 200), 135,70,43,28,22, and 17 kdaltons apparent molecular weights. Conditions for accurate determination of amanitin inhibition of the enzyme are established using ^{3}H-amanitin and are presented for the first time for plant RNA polymerases II; RNA polymerase II from these cultures is shown to be inhibited by 50% at 3-5 nM by α-amanitin, a value 10-50 times lower than previously reported.

Suspension cultures of carrot are shown to be inhibited more effectively by derivatives of α-amanitin (i.e., 6'-O-methyl-α-amanitin and 6'-deoxy-α-amanitin) that lack a 6'-hydroxyindole nucleus. Evidence from additional studies shows selective degradation of α-amanitin (AMA) compared to other derivatives; this degradation is postulated to be mediated by plant oxidases and occurs to the extent of 50% within 5 days.

Following studies to increase synthetic yields of 6'-O-methyl-α-amanitin, carrot cell lines resistant to this toxin were selected. Suspension cell cultures exposed to 20 μM inhibitor yielded two resistant lines with an apparent frequency of $5x10^{-7}$. These lines lacked the ability to degrade the selecting agent, and were thus similar to wild type. RNA polymerase II activities were indistinguishable from that obtained from wild type cells. Both lines exhibited an increase in RNA polymerase I levels of 25-40%.

The potential of using amanitin-based affinity ligands for retrieval of plant RNA polymerase II is described. Amanitin which has been linked to Concanavalin A and

2-iminobiotin retains inhibitory activity towards the enzyme. RNA polymerase II is shown to be fully active at the higher pH needed for the avidin-iminobiotin interaction. These systems are discussed relative to the potential for single step purification of RNA polymerase II and that of estimating contact radii of RNA polymerase II and avidin.

SECTION I

OBJECTIVES AND BACKGROUND

Objectives

The overall objectives of these studies were the selection and characterization of amanitin-resistant cell lines of higher plants using carrot as a model system. The specific means to achieve these objectives were the following: 1) Evaluate cellular and RNA polymerase II sensitivities toward amanitin derivatives for the selection of inhibitor-resistant, stable, genetically-defined lines of Daucus carota. 2) Characterize such mutants derived from selection and determine the nature(s) of resistance to the selected inhibitor. 3) Devise affinity methods for the purification of amanitin-binding proteins in wild type and mutant cells (i.e., RNA polymerases II and III and other proteins conferring resistance).

Background

Nuclear DNA-Dependent RNA Polymerases

Our present perception of the transcriptional elicitation of information from DNA in eukaryotes is that this process is mediated by at least three distinct DNA-dependent

RNA polymerases. These three classes of enzymes, denoted as I, II, or III, are believed to be responsible for the discrete synthesis of rRNA, hnRNA and some snRNA, and 5S and tRNA transcripts, respectively, in nuclei (reviewed in Roeder, 1976; Zieve, 1981). These enzymes may be further distinguished by their relative sensitivities toward the fungal cyclopeptide toxin, α-amanitin, in which concentrations for 50% inhibition of *in vitro* transcription are presently considered for plant enzymes to be 10-50 nM and 2.5 μM for classes II and III, respectively, with RNA polymerase I apparently insensitive at concentrations exceeding 1 mM (Guilfoyle, 1981; Jendrisak, 1981). Additionally, these enzymes have been described relative to their preferences for divalent cations (Mg vs. Mn), templates (poly(dAdT) vs. DNA), and ammonium sulfate optima (Roeder, 1976), although it remains to be seen whether such differences are significant *in vivo*.

The purification of these enzymes is generally achieved through a combination of methods including solubilization, adsorption and elution from polyethylenimine and chromatography on resins such as DEAE-substituted matrices, phosphocellulose, DNA-agarose, and heparin-agarose (reviewed by Lewis and Burgess, 1982). The resolution of each class of RNA polymerase from the others may exploit the differential binding to polyethylenimine, but usually is achieved by chromatography on DEAE resins. In this case, an overlapping position of elution is seen with DEAE-Sephadex (in which I

is resolved from II and III) and DEAE-cellulose (I and III are resolved from II), such that the appropriate combination may be exploited to achieve the purification of a specific class of enzyme. Subsequent purification by chromatography on the resins listed above or by sucrose gradient centrifugation usually yields a homogeneous enzyme.

Apparent subunits that copurify with the nuclear RNA polymerases demonstrate the complexity of these enzymes compared to the prokaryotic counterparts (reviewed in Paule, 1981). Holoenzyme from E. coli contains a core composed of β' (165 kd), β (155 kd), and α (39 kd), with σ (90 kd) completing the holoenzyme. In contrast, nuclear RNA polymerases contain 10-14 associated proteins, some showing apparent conservation between each class (Guilfoyle, 1981). Table I-1 demonstrates this complexity for plant enzymes, although similar comparisons can be made among animal and yeast enzymes. This analysis shows that the enzymes generally have unique subunits of molecular weights greater than 40 kilodaltons, while some subunits of lower molecular weight share structural homologies among the classes. Presumably, the discrimination between gene classes is governed by the larger subunits, and the common small subunits may specify some shared feature required for transcription of all three classes of enzyme.

Table I-1. Putative Subunit Structures for Plant Nuclear RNA Polymerases.

Cauliflower			Wheat Germ		
I	II	III	I	II	III
190	180		200	220	
		155		140	145
128	138	135	120		135
		90			90
		70	50		52
45	40	50	38	42,40	38
25	25	25	32		30
22	22	22	24	27,25	28,25
	19		21	21,20	22,21
17.5	17.5	17.5	17.8	17.8	17.8
17	17	17	17	17	17
	16.5,16			16.3,16.0	
	14			14	

a. Molecular weights in kilodaltons.
b. Underlined subunits immunologically cross-reactive.
c. Taken from Guilfoyle, 1981.

Structure and Function of Plant RNA Polymerases II

RNA polymerase II as purified by methods described above from cauliflower and wheat germ displays the subunits shown in Table I-1. Although some differences between wheat germ and cauliflower enzymes are apparent in the 27 kilodalton and 40 kilodalton proteins, one very obvious difference is that in the largest subunits. In this case, rapidly proliferating tissue such as cauliflower appears to have a large subunit of 180 kilodaltons, while the largest in wheat germ is 220 kilodaltons. This heterogeneity is also known for animal RNA polymerases II (Lewis and Burgess, 1982) and, as analyzed in plant systems, is considered to be an artifact. Early studies by Guilfoyle and Jendrisak (1978) showed that enzyme from rapidly proliferating tissues contains a 180 kilodalton subunit whereas that from quiescent tissue (wheat germ) contains mainly the 220 kilodalton subunit. These authors showed the presence of a protease activity in the former that could cleave the 220 kilodalton subunit to yield a subunit of 180 kilodaltons; the association of a structural change in the enzyme with the proliferating nature of the tissue was considered significant. Guilfoyle et al. (1984) have shown through examination of the enzyme structure by immunoblotting however, that this proteolysis occurs during purification, and conclude this difference to be an artifact of purification.

Although a consensus subunit profile may be observed for RNA polymerases II among many plant species, the

validity of these proteins as "subunits" will ultimately require an ascription of function. Presently, structure-function relationships are known only for the two largest subunits of RNA polymerase II. Bateman and Nicholson (1984) have shown the proximity of these two largest subunits (220 and 140 kilodaltons) by using reversible crosslinkers with wheat germ RNA polymerase II and suggest these two proteins may form a "core" around which the other subunits are arranged. Monoclonal antibodies to the largest subunit affect DNA binding (Carroll and Stollar, 1983) and template specificity for *in vitro* adenovivus transcription (Dahmus and Kedinger, 1983) by calf thymus enzyme; this suggests that the largest subunit of polymerase II is intimately involved in template selection. Additional studies using radiolabeled substrates for initiation and elongation (Cho and Kimball, 1982; Cho *et al.*, 1982) implicate the 220 kilodalton and 140 kilodalton subunits with initiation and elongation, respectively. Lastly, the largest subunit is known to be altered in *Drosophila* amanitin-resistant RNA polymerase II (Greenleaf, 1983); in conjunction with the studies of Brodner and Wieland (1976) showing covalent attachment of tritiated amatoxin to the 140 kilodalton subunit of enzyme from calf thymus, these results localize regions of the enzyme which contact the amatoxins. To date, functions for other subunits comprising RNA polymerase II are not known.

In Vivo and In Vitro Transcription by Plant RNA Polymerases II

Although the initial studies implicating classes of DNA-dependent RNA polymerases with transcription of groups of genes were accomplished in animal cells, subsequent studies in plants showed the existence of analogous enzymes functioning in a similar manner. Seitz and Seitz (1971) demonstrated that callus cells of parsley were sensitive to α-amanitin and that prolonged exposures to such would reduce poly A^+ rich RNA levels; higher concentrations were then shown to eliminate all but ribosomal RNA synthesis, thus paralleling the sensitivity of isolated RNA polymerases I and II with in vivo capabilities. Gurley et al. (1976) convincingly demonstrated that RNA polymerase I-rich chromatin from soybean would preferentially transcribe rRNA genes and was thus analogous to the animal enzyme in its properties. In a study of transcription in isolated nuclei from wheat, Luthe and Quatrano (1980) examined labeled RNA fractionated on sucrose gradients. By examining profiles produced under different ionic strengths, α-amanitin concentrations, and with divalent cations, the authors concluded that these nuclei had the capacity for synthesis of hnRNA, rRNA and species possibly synthesized by RNA polymerase III. These and other studies described below provide cogent arguments linking the synthesis of RNA classes (i.e., ribosomal, transfer RNA) with discrete RNA polymerases.

A series of studies have investigated polymerases involved in expression of T-DNA sequences in plants. The

known ability of this T-DNA (or Tumor-inducing DNA) to integrate into plant DNA sequences following transfer from Agrobacterium provides an attractive system to study transcription in plants (reviewed in Chilton, 1982). Beiderbeck (1972) showed that the induction of tumors by Agrobacterium on Kalanchoe leaves was sensitive to α-amanitin, if the addition of this inhibitor was made within 24 h of inoculation; that this was true, despite the fact that the bacterium was insensitive to α-amanitin, provided strong evidence that host RNA synthesis was required for tumorigenesis. Recently, Willmitzer et al. (1981) examined the expression of T-DNA transcripts in nopaline and octopine tumor lines of tobacco. Isolated nuclei derived from tumor lines were shown by Southern blotting to synthesize transcripts whose appearance was sensitive to low concentrations of α-amanitin; since these same transcripts were known to be polyadenylated, the involvement of RNA polymerase II in the transcription of genes transferred by the bacterium was inferred.

An additional model system for the study of transcription in plants is provided by cauliflower mosaic virus (CaMV). This double-stranded DNA virus appears to replicate in nuclei as a minichromosome similar to SV40 (Olszewski et al., 1982) and also codes for several transcripts (Pfeiffer and Hohn, 1983) including those for inclusion body proteins and aphid transmission. Guilfoyle (1980) examined expression of transcripts in turnip infected with CaMV in vivo and

in vitro; transcripts produced using purified nuclei hybridized to the same CaMV DNA strand as those produced in vivo. Further, the in vitro synthesis was sensitive to low levels of α-amanitin, and, in conjunction with ammonium sulfate optima seen, RNA polymerase II was thus concluded to be involved. Cooke et al. (1983) have proven this fact using purified RNA polymerase II to transcribe CaMV DNA on a plasmid. Previous attempts to achieve selective initiation of cloned templates with highly purified plant RNA polymerases have failed presumably because essential transcriptional factors are removed during polymerase purification as in the case of animal polymerases II (reviewed in Dignam et al., 1983; Cooke et al., 1983). However these workers primed RNA synthesis with a dinucleotide monophosphate (ApG) and ATP and were able to demonstrate that transcripts hybridizing only to the CaMV insert were produced by RNA polymerase II.

A series of experiments by Muhlbach and Sanger (1979) and those of Rackwitz et al. (1981) have provided evidence for the astonishing association of RNA polymerase II with viroid replication. Viroids, which are small (360 nt) single stranded circular RNA molecules that cause infectious diseases in plants, were postulated to be replicated either by RNA-dependent RNA polymerases (known to occur in higher plants) or by reverse transcriptases, which were not known to occur in plants (Diener, 1979). Muhlbach and Sanger (1979) showed that the production of viroid RNA, but not

cellular rRNA nor tRNA, in cucumber pale fruit viroid (CPFV)-infected protoplasts was inhibited more than 75% in the presence of 50 µg/ml α-amanitin; their observation that viroid RNA synthesis was also affected by actinomycin D prompted them to conclude that an RNA-dependent RNA polymerase was not involved in replication of this viroid. Rackwitz et al. (1981) then provided the most convincing evidence that RNA polymerase II was involved in viroid replication. These investigators showed that purified RNA polymerase II from tomato or wheat germ could provide in vitro full length transcripts when offered a variety of viroid templates. The binding of iodinated viroid RNA to RNA polymerase II, its demonstrated ability to compete with DNA for the template binding site, and inhibit DNA-dependent RNA synthesis by the enzyme, further established the feasibility of such a replication role for RNA polymerase II and suggested a mechanism for pathogenesis.

Additional processes in plants, where RNA polymerase activities have been examined, include those involving growth and development. Germination of wheat embryos was shown to be inhibited by very low concentrations of α-amanitin in a manner paralleling the in vitro inhibition of RNA polymerase II activities and that of in vivo synthesized poly A^+ RNA (Jendrisak, 1980). Since germination was also cycloheximide sensitive, it was obvious that both de novo mRNA synthesis and protein synthesis were critical components of germination. Increased levels of RNA

polymerases I and II are seen in seedling responses to auxin (Guilfoyle, 1980; Jacobsen, 1977) and also in germinating soybean axes (Guilfoyle and Malcolm, 1980). Although specific activities are changed there is no increase in polymerase protein nor any change in modification or polypeptide composition (SDS gels), with the exception of a cleavage of the largest subunit (215 kilodaltons) of RNA polymerase II to a smaller size (180 kilodaltons). The significance of this change is uncertain, and the differences in transcriptional patterns during these times may likely reflect an alteration of polymerase activity by stimulatory factors or other mechanisms.

Genetics and Regulation of Eukaryotic Nuclear DNA-Dependent RNA Polymerases II

Mutants of eukaryotic RNA polymerase II have been isolated using two approaches. One approach is to exhaustively screen temperature sensitive cells for RNA synthesis. This method, used by Winsor et al. (1979), yielded an RNA polymerase II mutant in yeast that had altered rates of RNA initiation and elongation when cells were grown at the nonpermissive temperature. Subsequent characterization of this enzyme (Ruet et al., 1980) showed a correlation of defective RNA synthesis and DNA binding with changes in the peptide map of the largest subunit. They further demonstrated that the purified enzyme lacked two smaller molecular weight subunits present in the wild type enzyme.

Although this approach provided worthwhile basic information, it is inconvenient when compared to the use of selective inhibitors.

The aforementioned inhibitory property of α-amanitin toward RNA polymerases II has recommended its use in the selection of mutants altered in such enzymes. The interaction of this cyclic peptide with mammalian RNA polymerase II is exceptionally tight, with K_D values being 10^{-9} M to 10^{-11} M (Cochet-Meilhac and Chambon, 1974). This inhibitor is known to bind in a 1:1 stoichiometry with polymerase in a manner independent of the availability of template or substrates. Further characterization has shown that the inhibition in vitro does not release template, RNA, or substrate but instead immobilizes the enzyme in a ternary complex with template and RNA. The specific step blocked in RNA synthesis is not initiation, but rather the polymerization of the second phosphodiester bond, or elongation (Cochet-Meilhac and Chambon, 1974; Vaisius and Wieland, 1982).

The first isolation of α-amanitin-resistant RNA polymerase II mutants was reported by Chan et al. (1972), who selected for CHO cell lines that grew in normally inhibitory concentrations of inhibitor. One clone which was characterized showed an amanitin-resistant activity eluting from DEAE-cellulose at a position corresponding to class II DNA-dependent RNA polymerase. No further characterization of either enzyme or cellular phenotype was reported at this

time. Subsequent mutants isolated by other workers have shown the temperature sensitive nature of such resistant cell lines and enzymes (Ingles, 1978), and demonstrated the codominant nature of resistance to α-amanitin (Lobban and Siminovitch, 1975). The definitive proof that such mutants had altered binding properties for labeled α-amanitin was shown by Crerar et al. (1977).

The regulation of synthesis and degradation of RNA polymerase II was explored in CHO cells heterozygous for α-amanitin resistance. Guialis et al. (1977) showed that cells (originally containing a 1:1 mixture of sensitive and resistant RNA polymerases II) grown in α-amanitin were able to inactivate the sensitive enzyme with the corresponding appearance of a proportional amount of resistant RNA polymerase II. Since no change in enzyme protein occurred during this induction and because loss of labeled amanitin binding occurred with the appearance of resistant activity, these studies collectively suggested that the level of RNA polymerase II in cells was maintained by an absolute concentration of the enzyme, and, further, that this regulation may be autogenous. Guialis et al. (1979) extended such studies to examine the subunit composition of RNA polymerase II from these lines during α-amanitin induction. By examining ^{35}S-methionine incorporation into polymerase peptides, they concluded that degradation of sensitive enzyme involved at least the two largest subunits of the enzyme, while synthesis of resistant activity showed incorporation of

label into these subunits as well as proteins of 16.5, 23, and 34 kilodaltons. The generality of this regulation was additionally confirmed in rat myoblasts (Crerar and Pearson, 1977).

The extension of α-amanitin resistant mutants to whole organisms began with the isolation of a Drosophila mutant tolerant of α-amanitin concentrations that were lethal to wild type (Greenleaf et al., 1979). This mutant also possessed altered RNA polymerase II activities with an apparent difference in 50% inhibition compared to the wild type of 250-fold. This lesion was demonstrated to map to the X chromosome, and heterozygous resistant organisms showed biphasic RNA polymerase II activity profiles analogous to cell line mutants (Greenleaf et al., 1980). Subsequent characterization of the mutant enzyme revealed a lower activity with Mg^{2+} as divalent cation and a reduced affinity for UTP and GTP (Coulter and Greenleaf, 1982). As described above, this mutation is in the largest subunit of RNA polymerase II (Greenleaf, 1983). The recent report by Sanford et al. (1983) of an altered RNA polymerase II activity in Caenorhabditis represents the only other whole organism reported to date with a demonstrated bona fide α-amanitin resistant RNA polymerase II.

Genetic studies on RNA polymerase II are few at present. Searles et al. (1982) were able to isolate a genomic fragment of Drosophila DNA that confers α-amanitin resistance by using transposon tagging. Subclones of this

DNA representing 14 kb of DNA were subsequently shown to code for several transcripts in Drosophila, one of which (7 kb) was shown to hybridize with mammalian sequences that were acquired in recipient cells when DNA from amanitin-resistant cells was used for transformation (Ingles et al., 1983). The latter experiment exploited the extensive structural conservation of RNA polymerases mentioned above. Sequences encoding this transcript were subcloned, inserted into a bacterial plasmid suitable for hybrid protein production, and shown to code for the largest subunit as assessed by subunit-specific antibody probes (Greenleaf, 1983). Other transcripts originating from the subclones above apparently do not encode Drosophila RNA polymerase II subunits (Arno Greenleaf, personal communication).

A second and novel approach to isolating genes coding for RNA polymerase II subunits has been accomplished in yeast (Young and Davis, 1983). These workers probed a library of yeast DNA constructed in a vector yielding high levels of hybrid protein and selected several colonies on nitrocellulose filters with polyvalent antibodies to RNA polymerase II. An examination of the colonies with subunit-specific antibody identified clones corresponding to the two largest subunits of the enzyme; Southern analysis demonstrated genes for those two to be in single copy for the haploid genome of yeast. Genes for the other putative subunits of yeast RNA polymerase II have been cloned using antibody specific to these as well. Experiments in which

the genes for the two largest subunits are deleted show these subunits to be essential components of the enzyme since lethal phenotypes result (R. Young, personal communication).

Phenotypes of Mutant RNA Polymerases II

Not unlike their procaryotic counterparts, mutants in RNA polymerase in eukaryotes have been seen to possess phenotypes traceable to an altered enzyme. Greenleaf et al. (1980) have shown in collaboration with Mortin and Lefevre (1981) that mutations in RNA polymerase II confer developmental abnormalities to Drosophila flies. One mutation (Ubl), shown to be allelic with α-amanitin resistance, produced flies with enlarged capitella having more bristles than wild type. Crerar et al. (1983) observed differences in myogenic differentiation of rat myoblasts possessing α-amanitin-resistant RNA polymerases II. Where wild type cells could be induced to form multinucleate myotubes in culture, the heterozygous mutants when grown in α-amanitin appeared to be myogenic-defective and produced low levels of muscle creatine kinase and muscle proteins (myosin, troponin). The collective conclusions to be drawn from these and the Drosophila studies were that mutants with alterations in RNA polymerase II could be acquired that undergo normal proliferation but altered differentiation, and that developmental genes may be recognized differently by RNA polymerase II than are other genes.

Rationale

The ability to introduce a defined mutation into plant RNA polymerase II will better define the roles of this enzyme in developmental gene transcription, nuclear-plastid interactions, viroid and CaMV replication, expression of foreign genes such as those encoded by T-DNA and other processes. The defined mutations in RNA polymerase II generated with α-amanitin thus merit its use as a selecting agent for plant cell mutants. A study of the factors affecting the response of carrot cells and RNA polymerase II to various derivatives of α-amanitin will be undertaken to assist in choosing a derivative suitable for the selection of mutants. The appropriate derivative will then be used to select for cells capable of surviving lethal doses of toxin, following an exposure to mutagen. Cell lines thus obtained will be examined for abilities to survive exposure to toxin through altered RNA polymerase II or capacity to degrade the selecting agent. The screening for RNA polymerase II mutants will utilize assays of crude activities and fractions enriched for polymerase II derived from cells grown in the absence and presence of α-amanitin.

To assist in discriminating the bases of resistance, the purification of α-amanitin binding proteins will be useful. Thus, proteins such as transport molecules, degradative enzymes, and altered RNA polymerases II may be examined. The construction of α-amanitin-based affinity ligands for this purpose will be undertaken. These ligands

will be sought by covalently linking α-amanitin to soluble entities, which possess retrievable determinants. One approach will be to link α-amanitin to Concanavalin A and exploit the elution by glucose of the complex with RNA polymerase II from Sephadex. The second approach will be to couple α-amanitin through a spacer to 2-iminobiotin and attempt elution of RNA polymerase II bound to amanitin from avidin-agarose. Aside from purifying α-amanitin-binding proteins in general, the ligands thus obtained may help define the complete spectrum of proteins participating in transcription by RNA polymerases II and III.

SECTION II

PREPARATION AND PURIFICATION OF METHYLAMANITINS

Introduction

Wieland and Fahrmeir (1970) first demonstrated that α-amanitin (AMA) can be converted to 6'-O-methyl-α-amanitin (meAMA) by reaction with diazomethane. The methylated AMA, which could be purified by chromatography on Sephadex LH20, is easily characterized by observing the loss of a 20 nm bathochromic shift when ultraviolet spectra are recorded at pH 11 or greater (Faulstich et al., 1981). This methyl group is needed to protect the labile indole nucleus of AMA, which contains a 6'-hydroxylindole moeity, from oxidations by $NaIO_4$ or tyrosinase enzymes (Wieland, 1983; Romeo and Preston, 1984).

My need for milligram amounts of meAMA for experiments in subsequent sections prompted my synthesis of several preparations. The yields in these preparations were not unlike those of other preparations from our laboratory, in that both the yields of meAMA and the recoveries of starting material were quite variable from one synthesis to the next. Since the selection of resistant plant cell lines could potentially require 50-100 mg of meAMA (for reasons which

will become obvious later), an examination of the factors that might optimize the yields of this derivative was undertaken.

It is shown below that yields of meAMA can be controlled by careful attention to the amount of diazomethane that is used and that this reaction can be comfortably scaled up to allow the synthesis of large (50-100 mg) amounts of meAMA. Since meAMA and AMA are shown to be readily resolved by semipreparative hplc the time requirement for synthesis and purification can be 1 day or less. Finally, the identity of a major, but controllable, by-product of this methylation is established to be the potentially useful O,N-dimethyl-α-amanitin.

Materials and Methods

Reagents and Chemicals

α-Amanitin (AMA) was a product of our laboratory and purified as described (Little and Preston, 1984). Solvents used for chromatography were hplc grade (Fisher), Diazald and d_6-DMSO (100 atom %) were from Aldrich and sample tubes for NMR were purchased from Wilmad Glass (528p).

Generation and Titration of Diazomethane

Diazomethane (CH_2N_2) was produced in a diazomethane generator (Pierce Chemical Co.) using a modification of the procedure in Fieser & Fieser (1967). The modification used

was the addition of 5 ml ethanol to the KOH in the distilling flask prior to the delivery of the ethereal Diazald solution. This is apparently an omission from the published procedure, and the ethanol was found to be necessary for the reaction to occur. Freshly distilled solutions of CH_2N_2, which were maintained on ice until use, were transferred with flame-polished pipets (due to the explosive nature of this gas with ground glass surfaces; see Fieser and Fieser (1967)).

The estimation of CH_2N_2 concentrations in distillates was made with a modification of the method listed in Fieser and Fieser (1967) Ten ml 0.1 M benzoic acid in methanol were reacted at room temperature with 2 ml distillate. After 30 min., 1.0 ml of the mixture was diluted with 100 ml water and titrated to the equivalence pH (7.59 in this case) with 0.1 M NaOH. Diazomethane prepared as above and titrated in this manner was typically 0.06 M to 0.12 M.

Reaction of Diazomethane with α-Amanitin

The assessment of CH_2N_2/AMA ratios to use for optimal synthesis of meAMA was done on a microscale as follows. To individual tubes (16x150 mm with teflon screw caps) each containing 5 μmoles AMA in 2 ml methanol (on ice), was added 0.2 to 2.0 ml of CH_2N_2 solution, cap, and store at room temperature. After 30 min., a stream of nitrogen gas is delivered to the tubes within a fume hood and the solutions taken to dryness. The dried material was redissolved in

running solvent and subjected to hplc. Peak heights derived from constant volume injections were used to calibrate reaction yields.

Preparative syntheses of meAMA were made as above using 20 moles CH_2N_2 per mole AMA. In instances where ether from the CH_2N_2 solution began to precipitate AMA from the methanolic solution, additional methanol was added. Since volumes thus varied, the substrate concentration products for these reactions were 50 $(mM)^2$ to 90$(mM)^2$. Removal of unreacted CH_2N_2 and product purification by hplc was as above. Verification of the structure of meAMA was confirmed by loss of the 6'-hydroxyl using ultraviolet spectroscopy (in base) and by comparison to NMR spectral assignments (Wieland et al., 1983).

High Performance Liquid Chromatography

The separation of AMA, meAMA and dimeAMA by high performance liquid chromatography (hplc) was carried out on a Zorbax C18 semipreparative column eluted with 19% acetonitrile in water using conditions as described (Little and Preston, 1984). Both water (deionized and glass-distilled) and sample were prefiltered through a 0.2 μ filter (Millipore) before use.

Spectroscopy

Ultraviolet spectra were recorded on a Beckman 25 recording spectrophotometer. Dr. Lester Taylor, Dept.

Chemistry, Indiana University, Bloomington, provided fast atom bombardment mass spectra (FABMS) on samples reconstituted in glycerol: oxalic acid (1:1).

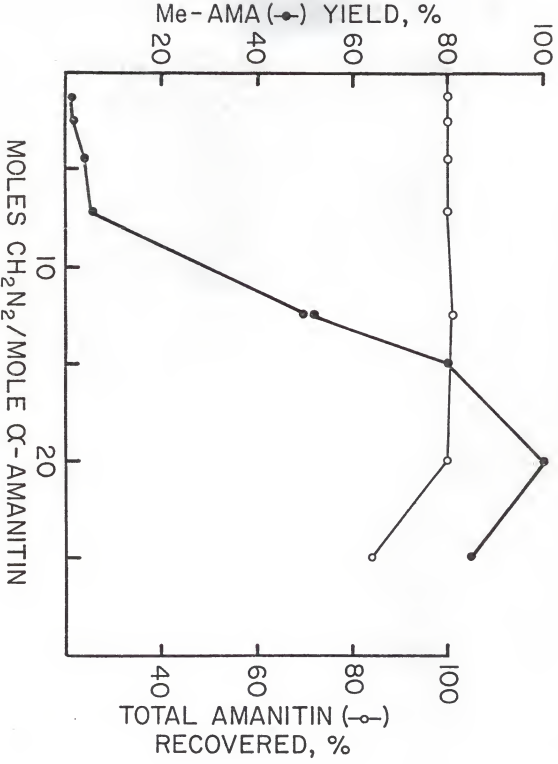
Proton magnetic resonance spectra (NMR) were kindly provided by Ms. Sandra Bonetti and J.E. Gander, Dept. Microbiology and Cell Science, University of Florida. Samples which were taken to dryness in 4 ml glass vials were treated with several 0.5 ml rinses of absolute ethanol (to eliminate traces of water) which were removed in vacuo. Samples (5 μmoles) were dissolved in 0.5 ml d_6-DMSO containing 1% TMS and spectra acquired on a Nicolet NT-300 operating at 300 Mhz in the FT mode. To ensure the detection of slowly relaxing nuclei, 10 second delays interrupted the pulses (7 μsec).

Results

Determination of Conditions for Optimal Yields of meAMA

Figure II-1 depicts the results of the microscale syntheses in which molar ratios of CH_2N_2/AMA were varied. An examination of this figure shows that the recovery of meAMA is a function of this ratio, as is the recovery of product and reactant after reaction (AMA and meAMA totals). The arithmetic products of reactants were 24 to 40 $(mM)^2$. Under these conditions, approximately 20 moles CH_2N_2/mole AMA appears optimal.

Figure II-1. Yields and Recoveries of Amanitins Treated With Diazomethane. The recoveries (open circles) of AMA and meAMA along with yields (closed circles) of meAMA that are achieved by varying the initial ratio of CH_2N_2 to AMA are compiled. Reaction conditions are as described in the text.


Me-AMA (-●-) YIELD, %
20
40
60
80
100
MOLES CH_2N_2/MOLE α-AMANITIN
10
20
TOTAL AMANITIN (-○-) RECOVERED, %
40
60
80
100

Preparative Syntheses of meAMA

Table II-1 describes the yields of meAMA and recoveries of meAMA and AMA in a series of four separate syntheses. In all cases, molar ratios of 20:1 (CH_2N_2:AMA) were used with some variation in the concentration products of the reactants, as listed in the table. The conditions established above provide a good yield of product at arithmetic concentration products at 56 $(mM)^2$.

Characterization of O,N,-Dimethylamanitin

The fractionation by hplc of a typical preparative synthesis (Reaction 1, Table II-1) of meAMA is given in Figure II-2. Positions A,B, and C are AMA, meAMA, and dimeAMA (O,N,-dimethylamanitin) respectively. The peak of dimeAMA was never before seen in these syntheses; it appears in greater quantity in those reactions where the optimal level of CH_2N_2 (relative to meAMA yields) has been exceeded.

The identity of peak C was established by a combination of methods. First, an increased hydrophobicity was suggested by its retention on a reverse phase column (Figure II-2). This was supported by ultraviolet spectroscopy. Figure II-3 shows that the UV spectrum of this yellow compound (pure by tlc and hplc, not shown) has experienced a 3 nm bathochromic shift. Spectra run at pH 12 (not shown) demonstrated the presence of a 6'-methoxy derivative similar to meAMA. Hence, a O,N-dimethylamanitin was postulated.

Table II-1. Tabulated Yields of MeAMA and AMA Recovered from Preparative Methylations.

Reaction	Initial AMA, μmoles	CH_2N_2 x AMA $(mM)^2$	AMA Yields μmoles	meAMA Yield μmoles (% Yield)	Total % Recovery
1	86	98	1.1	54 (64%)	64%
2	26	98	2.7	14 (62%)	64%
3	35	98	0.4	16 (47%)	47%
4	65	56	0.4	62 (95%)	95%

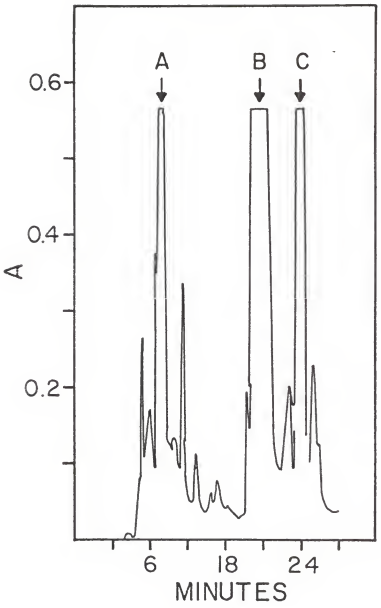


Figure II-2. Preparative Purification of Methylation Reaction Products by Reverse Phase HPLC. The fractionation of a crude reaction mixture by reverse phase hplc yields AMA (A), meAMA (B), and dimeAMA (C). Absorbance of the elute at 304 nm was monitored.

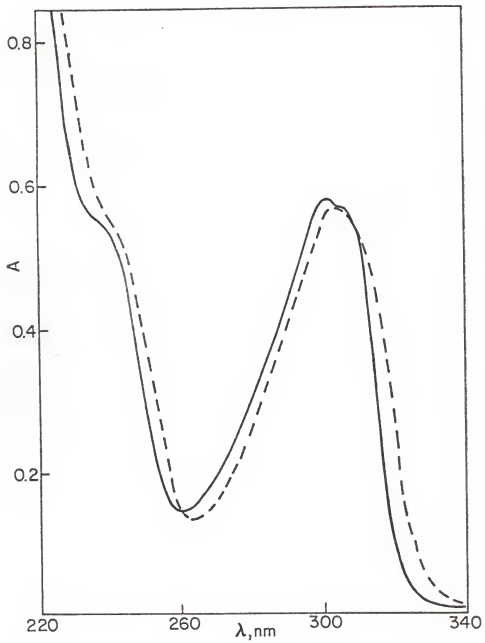


Figure II-3. Ultraviolet Spectra of Methylated Amanitins. Ultraviolet spectra in water (pH 7) are presented for meAMA (solid line) and dimeAMA (dotted line).

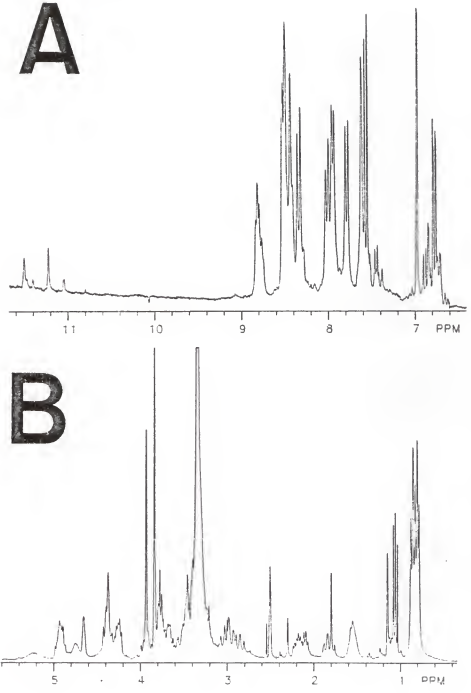
A comparison of the ^{1}H-NMR of this compound (Figure II-4) with authentic meAMA showed two obvious differences. First, the indolyl NH of meAMA at 11.4 ppm was absent. Second a sharp singlet at 3.928 ppm, that was not coupled to other nuclei (not shown), was acquired in this compound. The absence of splitting in the latter peak and the chemical shift suggested a methyl substituent on a heteroatom. The simultaneous loss of the indole NH provided the strongest clue that the substitution was on this heteroatom.

The unequivocal nature of the proposed structure was established by FABMS. Table II-2 compares calculated and experimental values for the quasimolecular ion, $M+H^{+}$, and the corresponding sodium adduct, $M+Na^{+}$. From these data one can infer an observed mass of 946.22 daltons, an increase of 14 AMU over that of meAMA. The odd nitrogen rule (McLafferty, 1973) requires molecules containing an even number of nitrogens to have an even mass (M^{+}); since meAMA

Table II-2. Comparison of FABMS Calculated and Observed Masses.

Mass	$M+H^{+}$	$M+Na^{+}$
Calculated	947.35	970.34
Observed	947.22	970.22

Figure II-4. Proton NMR Spectra of O,N-Dimethyamanitin. Proton magnetic resonance (NMR) spectra were recorded as described in the text on hplc purified dimeAMA. Figure II-4a shows the loss of the peaks at 9.1 ppm and 11.4 ppm which represent the indole 6'-hydroxyl and NH respectively. Figure II-4b shows peaks at 3.834 ppm and 3.928 ppm representing the methyl signals on the indole 6'-hydroxyl and NH respectively.


A
11
10
9
8
7
PPM
B
5
4
3
2
1
PPM

already possesses 10 nitrogens and since the observed mass, 946, is itself even, the 14 AMU increase cannot be a result of acquisition of a nitrogen atom from the CH_2N_2. The only possibility is the addition of CH_2, which, substituted onto NH to give N-CH_3, would explain not only the 14 AMU difference between this compound and meAMA, but the NMR and UV spectra as well.

Discussion

The relationship of the desired product meAMA to ratios of CH_2N_2/AMA provides a rational explanation for varied yields seen in other preparations made in this lab. Perhaps most surprising was that a 20:1 ratio was required for quantitative methylation, where lower amounts were insufficient. Extending the reaction time to 2 hours neither reduced product yields nor the ratio requirement for conversion (not shown). The ability to scale this reaction up for preparation of larger amounts of meAMA is shown in Table II-1. In this case, recoveries are as expected when concentration products are appropriately maintained. Variables which might affect this, from one batch of CH_2N_2 or AMA to the next, might also include the amount of water codistilling with the CH_2N_2 and the purity of the AMA. The latter is reflected in the yields (Table II-1) achieved when comparing reactions 1 and 2 with reaction 3. Although these methylations were effected from a common batch of CH_2N_2 and using identical ratios and concentration products, reactions

1 and 2 used AMA preparations of lower purity (A^{304}/A^{262} = 2.7 to 3.6) than that for reaction 3 (A^{304}/A^{262} = 3.9); impurities in the preparations may thus have competed for CH_2N_2 and raised the yields over that for very pure AMA, when CH_2N_2 was present in levels far greater than needed for optimal yield of meAMA.

The discovery that reactant conditions could be manipulated to optimize the synthesis of the desired meAMA was coincidental with the observation of a side product of this reaction. This yellow-colored product, which gave cinnamaldehyde-HCl reactivity similar to AMA (Preston et al., 1975) and an observed K_I (not shown) toward wheat germ RNA polymerase II of 5 nm, was unequivocally established as the O,N-dimethylated derivative of AMA. Faulstich et al. (1981) reported CD and partial UV data for a postulated O,N,-dimethyl AMA which appeared as an uncontrollable side-product when AMA was methylated with methyl iodide and sodium ethoxide. They found only slightly altered affinity of this dimethylated adduct for Drosophila RNA polymerase II.

In conclusion, the studies reported here have defined some relationships concerning the methylation of AMA. It is further established that the synthesis of meAMA can be optimized to give yields much higher than that with methyl iodide (30%; Faulstich et al., 1981); the scale up of this reaction to AMA amounts approaching the hundred micromole level can be easily accomplished, although it is recommended

that a microscale titration of a particular CH_2N_2 batch with AMA be undertaken first. Finally, the discovery that dimeAMA can be produced by this reaction provides us with an additional derivative that, because of its greater hydrophobicity (hence, more likely to better penetrate cells) and unaltered K_I for plant RNA polymerase II, may prove useful for selecting amanitin-resistant cell lines, perhaps even at doses lower than needed for meAMA.

SECTION III

PURIFICATION OF CARROT RNA POLYMERASE II AND INTERACTION WITH AMATOXINS

Introduction

In order to define the subunit composition of plant RNA polymerases II a number of purification schemes have been devised (reviewed in Guilfoyle, 1981). Contemporary approaches that yield homogeneous enzyme in 2-3 days generally follow enrichment on Polymin P (polyethylenimine), chromatography on DEAE-substituted resins, and affinity chromatography on phosphocellulose or DNA-agarose. Using these approaches, milligram quantities of polymerase II have been obtained from a variety of plants including wheat germ, rye, soybean, and cauliflower (Guilfoyle and Jendrisak, 1978; Jendrisak and Burgess, 1975). Such preparations have been used to examine both the subunit composition as well as α-amanitin sensitivities of these enzymes relative to class I and III RNA polymerases and the corresponding enzyme from animal sources (Jendrisak and Guilfoyle, 1978; Hodo and Blatti, 1977). Comparisons of class II RNA polymerases from plants have generally yielded common subunit features for these enzymes; in addition to two large subunits of 180-200 kd and 140 kd, there exist several subunits of 40 kd and

less. Recent experiments using crosslinking agents (Bateman and Nicholson, 1984) demonstrate that the two largest subunits are in close proximity to each other, while monoclonal antibody directed against the largest subunit blocks both DNA binding and specific transcription of adenovirus major late genes (Carroll and Stollar, 1983; Dahmus and Kedinger, 1983). Using radiolabeled affinity substrates that bind uniquely to these subunits, Cho and coworkers (Cho et al., 1982; Cho and Kimball, 1982) were able to show that catalytic sites, on wheat germ RNA polymerase II involved in initiation and elongation are, respectively, the 215 kd and 140 kd proteins. Thus, the most complete understanding to date is that, analogous to prokaryotic RNA polymerase, higher eukaryotic RNA polymerase II seems to possess a core enzyme of two adjacent high molecular weight proteins which appear capable of template binding and RNA polymerization.

A more complete understanding of plant RNA polymerases II may be gained by studying their interaction with α-amanitin. This fungal peptide is known to block transcription after the first nucleotide polymerization in vitro (Vaisius and Wieland, 1982) in a manner not competitively affecting nucleotide or DNA binding, nor release of RNA from the transcriptional complex (Cochet-Meilhac and Chambon, 1974). Since the 140 kd subunit of animal polymerases II may be covalently linked to ^{3}H-amanitin, and since Drosophila mutants resistant to α-amanitin have an altered 215 kd subunit (Brodner and Wieland, 1976; Greenleaf, 1983), the

site of amanitin interaction with the class II enzyme would appear to be the subunit core complex described above. As plant enzymes have been reported to possess subunits of analogous molecular weights, the molecular mechanistic studies and quest for phenotypes associated with amanitin resistance (see Section I) may be readily extended to higher plants.

The complete characterization of amanitin resistant mutants requires a comparison of mutant RNA polymerases II with wild type enzymes. Although all published reports of plant polymerases II relate the amanitin sensitivity of these enzymes, there is much variation in the absolute values of amanitin yielding 50% inhibition. A survey of papers shows such K_I values range from 50 to 250 nM for wheat germ RNA polymerase II (Jendrisak and Guilfoyle, 1978; Hodo and Blatti, 1977) and 70 nM for parsley RNA polymerase II (Link et al., 1978). Since most reports lack complete experimental detail, the reasons for these differences are uncertain, although three factors are likely: 1) inaccurate calibration of amanitins, 2) high concentrations of enzyme, and 3) incomplete binding of inhibitor to enzyme. For the purposes of comparison of wild type and amanitin-resistant polymerases II, as well as even considering the plausibility of selection of resistant mutants, RNA polymerase II has been purified from carrot suspension cultures. As I shall demonstrate, plant RNA polymerases II are at least tenfold more sensitive to amanitin than previously shown by kinetic

estimates, that such values may be confirmed by ^{3}H-amanitin binding, and that carrot enzyme is analogous in subunit structure to other plant RNA polymerases II.

Materials and Methods

Chemicals and Reagents

All reagents were analytical grade or better. Glass-distilled deionized water was used for the preparation of solutions. RNase-free sucrose and 5-[^{3}H]UTP (18 Ci/mmol) were from Schwarz Mann. Calf thymus native DNA, polyvinyl-pyrrolidone (PVP), bovine serum albumin (BSA) and phenyl-methyl sulfonyl fluoride (PMSF) were purchased from Sigma. Resins for chromatography were from Whatman except for ssDNA-agarose which was from P-L Biochemicals. Polymin P was a gift of Badische Anilin und Soda Fabrik WHOZ Haupt-laboratorium B9, Hochschullieferungen, 6700 Ludwigshafen/ Rhein Germany. Reagents for the preparation of gels were electrophoresis grade and were obtained from BioRad.

Buffers and Chromatography Resins.

Whatman DE52 and P-11 phosphocellulose were prepared according to manufacturers instructions and stored in buffer C. Buffers for isolation of RNA polymerase were buffer A (0.1 M Tris-HCl pH 7.9, 5°C, 0.25 M sucrose, 25 mM BME, 1 mM PMSF, 1mM EDTA, 0.5 mM $MgCl_2$); buffer B (0.05 M Tris-HCl pH 7.9, 1 mM EDTA, 5 mM BME, 1 mM PMSF); buffer C (0.05 M

Tris-HCl pH 7.9, 1 mM EDTA, 15 mM BME, 25% v/v glycerol). To prevent the apparent loss of thiol through oxidation by DMSO, BME was added just before buffer use. Stock solutions of PMSF (0.1 M) were in DMSO. A 10% solution of Polymin P was prepared as described by Jendrisak and Burgess (1975).

Cell Cultures.

Cultures of D. carota Camden Hybrid were derived from petiole sections of axenic plants and those of D. carota White Belgian were obtained from David E. Evans of DNA Plant Technology Corporation, Cinnaminson, NJ. Both cultures were maintained in MS salts medium supplemented with 3% sucrose, 0.2% casamino acids, 200 µg/L kinetin and 100 µg/L 2,4-D (Ammirato, 1983). For convenience in media preparation, 2,4-D and kinetin were prepared as the sodium and hydrochloride salts respectively by dissolving each in aqueous solutions containing one equivalent of sodium hydroxide or three equivalents HCl and lyophilizing the filtrates (0.2 µ filter). Callus cultures were maintained in the dark on medium supplemented with 1% agar while suspension cultures were maintained on a gyrotary shaker (New Brunswick G-10, 150 rpm) at 25°C, 3000 lux and 12:12 photoperiod.

Purification of RNA Polymerase II

Carrot RNA polymerase II was purified to near homogeneity following solubilization as described below. The

titration of the amount of Polymin P required to precipitate the enzyme was performed on Fr. 1 aliquots (of a separate batch of cells) that were previously adjusted to 0.075 M $(NH_4)_2SO_4$. Precipitates formed upon the addition of Polymin P were centrifuged as described below and supernatants assayed for protein and RNA polymerase activity as for wheat germ RNA polymerase II (Jendrisak and Burgess, 1975).

For the solubilization of RNA polymerase, suspension cultures in exponential phase were harvested on the 4th day, after transfer of 200 ml inoculum into 1 L of medium. Following collection on miracloth, the cells were rinsed with distilled water, packed into plastic bags, and frozen in liquid nitrogen. Cells (450 g) were quickly thawed in tap water, mixed with an equal volume of buffer A and ground to a slurry in a mortar and pestle. Following clump dispersion with a 15 s high speed homogenization in a Waring Blender, cells were passed once through a French Pressure Cell at 8,000-16,000 lb/in^2 and collected in a beaker containing 0.1 g PVP per gram cells. After stirring 10 min on ice, the slurry was centrifuged for 30 min at 10,000 x g and the supernatant collected via filtration through miracloth (Fr. 1). Following adjustment to 0.075 M $(NH_4)_2SO_4$ with 2 M $(NH_4)_2SO_4$, 10 µl of 10% Polymin P was added dropwise per ml of total volume and the mixture was stirred an additional 10 min. After centrifugation as above, the supernate was discarded and the pellet was resuspended with a Potter-Elvehjem mortar and pestle in buffer B plus

0.075 M $(NH_4)_2SO_4$ using 50 µl buffer per ml Fraction 1 total volume. After centrifugation as above, the supernate was discarded and the pellet resuspended in buffer B plus 0.25 M $(NH_4)_2SO_4$ as above and centrifuged. The pellet was discarded and protein was precipitated with 35 g $(NH_4)_2SO_4$ per 100 ml supernate. Following centrifugation at 14,000 x g for 20 min, the pellet was resuspended in buffer C to give a conductivity equal to buffer C plus 0.075 M $(NH_4)_2SO_4$ (Fr. 2).

Fraction 2 was applied to a 4 ml column of DE52 pre-equilibrated in the same buffer, washed through with 10 volumes of equilibration buffer and the polymerase was step eluted with buffer C plus 0.25 M $(NH_4)_2SO_4$. Fractions (1.2 ml) containing the bulk of activity were pooled, mixed with BSA and diluted with buffer C to 0.5 mg/ml BSA and 0.075 M $(NH_4)_2SO_4$ (Fr. 3).

Fraction 3 was applied to a 2 ml column of phosphocellulose that was pre-equilibrated with buffer C + 0.075 M $(NH_4)_2SO_4$ and 0.5 mg/ml BSA. Following a 10 volume wash of the same buffer the enzyme was eluted using buffer C + 0.25 M $(NH_4)_2SO_4$ + 0.5 mg/ml BSA in 1.2 ml fractions.

Pooled activity (Fr. 4) derived from phosphocellulose chromatography was diluted to 0.075 M $(NH_4)_2SO_4$ and 0.5 mg/ml BSA and loaded onto a 5 ml column of DNA-agarose equilibrated with buffer C + 0.075 M $(NH_4)_2SO_4$, washed through with 10 volumes of equilibration buffer lacking BSA and RNA polymerase was eluted with buffer C plus 0.3 M

$(NH_4)_2SO_4$. Individual fractions were frozen in liquid nitrogen. Wheat germ RNA polymerase II was purified using the method of Jendrisak and Burgess (1975).

Assay for Nonspecific Transcription and K_I Determinations.

RNA polymerase fractions were assayed at 25°C for 10 minutes using a transcription mix to provide the following components in a final volume of 100 µl: 0.05 M Tris-HCl pH 7.9, 3.3 mM $MnCl_2$, 8.3 mM KCl, 3.3 mM BME, 0.6 mM each of ATP, CTP, GTP, heat-denatured calf thymus DNA 160 µg/ml, and [^{3}H]UTP (2 Ci/mmole), 8 µM. Following 10 min incubation the reaction was stopped with 100 µl stop bath, 1 ml ice-cold 10% TCA was added and RNA was collected on GF/C filters and processed for counting as described for calf thymus RNA polymerase II (Preston et al., 1975). One unit of activity is that amount giving 1 pmole UMP incorporation in 10 min. Protein was assayed by the method of Bradford (1976).

Assays to determine K_I values were as described (Preston et al., 1975) with the following modifications. Enzyme (0.4 - 0.6 nM final) purified through DNA agarose was preincubated in 50 µl volume at 25°C with inhibitor. At the end of 30 minutes, 50 µl of transcription mix (Cochet-Meilhac and Chambon, 1974) was added and UMP incorporation followed for 10 min after which the reaction was stopped and processed as above. Control counts lacking inhibitor were typically 1000 cpm.

Polyacrylamide Gel Electrophoresis

RNA polymerase II purified through DNA agarose was analyzed on SDS denaturing gels of polyacrylamide (5 to 15% gradient gel) using a 3% stacking gel as described by Laemmli(1970). Two dimensional profiles of this same enzyme were derived by subjecting the enzyme to electrophoresis under nondenaturing conditions first (Sklar and Roeder 1976), with subsequent electrophoresis into a 10% polyacrylamide gel using the system of O'Farrell (1975). Following electrophoresis, gels were removed from the glass plates and stained for protein using the procedure of Wray et al. (1981). To minimize breakage, gels were transferred through staining solutions and washing using teflon screens (1000 μ mesh).

Amatoxin Preparation and Calibration

α-Amanitin (AMA) was a preparation of our laboratory and was purified as described previously (Little and Preston, 1984). The preparation and purification of 6'-O-methyl-α-amanitin (meAMA) is described in Section II. Amaninamide (deAMA), or 6'-deoxy-α-amanitin, was purified from methanolic extracts of Amanita virosa by sequential chromatography on Sephadex LH20 with 50% methanol, LH20 with 0.004 M NH_4OH (Buku et al., 1980) and reverse phase hplc (Little and Preston, 1984). Molar absorptivities used for quantitating amatoxins were 15,400 at 304 nm. For AMA and

meAMA (Cochet-Meilhac and Chambon, 1974) and 12,500 at 287 nm for deAMA (Faulstich and Wieland, 1968).

Binding of ^{3}H-Amanitin to RNA Polymerase II

Confirmation of kinetic estimates of K_I values was achieved using the binding of ^{3}H-O-methyl-dehydroxy methyl-α-amanitin to wheat germ RNA polymerase II. Periodate-oxidized α-amanitin was reduced with either $NaBH_4$ or $NaB^{3}H_4$ to provide this derivative as either radiolabeled or not, using the method of Wieland and Fahrmeir (1970). Over the course of these experiments, the radiolabeled deviative was repurified several times from radiolysis products using reverse phase hplc, and had a final specific activity of 11×10^{9} dpm/μmole.

The protocol and buffers used for binding were similar to those for calf thymus RNA polymerase II (Cochet-Meilhac and Chambon, 1974) except for the following modifications. All incubations were at 25°C in 13 x 100 mm polypropylene tubes. Unlabeled O-methyl-dehydroxymethyl-α-amanitin was added at the end of timed incubations (in order to provide a final concentration of unlabeled toxin 100 times that of ^{3}H-toxin) prior to placing tubes on ice. RNA polymerase-^{3}H-amanitin complexes that were collected on nitrocellulose filters (Millipore, HAWP, 0.25 μ) were quantitated by scintillation in Brays solution (Bray, 1960).

Results

RNA Polymerase II Purification

The conditions for complete adsorption of RNA polymerase II to Polymin P are given in Figure III-1. By normalizing the ratio of extraction buffer to cell mass, the concentration of protein was consistent from one preparation to the next. In this regard, complete adsorption of RNA polymerase II appears to occur at 2-4 µl Polymin P based upon soluble activity and protein. The apparent increase in protein content of the supernate and the absence of dramatic change in soluble activity are probably due to the difficulties of assay of protein and transcription under conditions where Polymin P is in excess. That is, Polymin P appears (unpublished) to interfere in the dye-binding assay for protein and may stimulate or inhibit transcription completely, depending on the concentration (hence the precipitation of the enzyme with $(NH_4)_2SO_4$ before chromatography). Thus, the value of 10 µl was chosen with the confidence that complete recovery was achieved (see also yields in Table III-1).

Figure III-2 depicts the gradient elution of RNA polymerase II from DE 52 and provides the basis for step elution in Figure III-3. The elution position here is at 0.15 M $(NH_4)_2SO_4$ where elution has been reported to occur at 0.22-0.25 M $(NH_4)_2SO_4$ (Jendrisak and Burgess, 1975). This is presumably caused by residual Polymin P after precipitation.

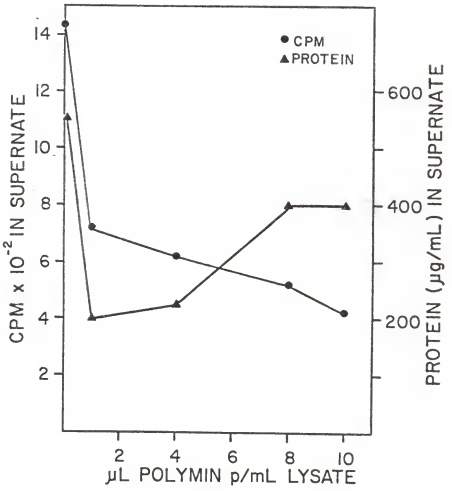


Figure III-1. Adsorption to Polymin P of RNA Polymerase II. Varying amounts of Polymin P were added to crude lysates of D. carota. After centrifugation as described in the text, the supernates were assayed for total RNA polymerase activity (closed circles) and protein (triangles).

Table III-1. Purification of RNA Polymerase II from D. carota Suspension Cells.

Fr.	Total Units	Units/mg Protein	Fold Purification	Yield %
1	11400	12.3	1	100
2	16100	-	-	141
3	16100	-	-	141
4	6300	-	-	55
5	2200	17045	1390	19

Note: Suspension cells (450 g) were disrupted to yield a crude extract (Fr. 1) which was precipitated with Polymin P and $(NH_4)_2SO_4$ (Fr. 2). Subsequent purification (fractions 3, 4, and 5) included DE52, phosphocellulose and DNA-agarose, respectively. One unit equals 1 pmole UMP incorporation in 10 minutes as described in the text.

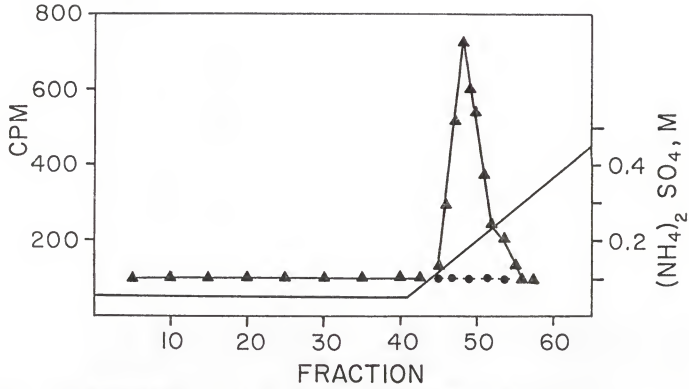


Figure III-2. Gradient Elution of Carrot RNA Polymerase II from DE-52. Ammonium sulfate pellet from Polymin P elute was diluted with buffer C to 0.075 M $(NH_4)_2SO_4$ and applied to a column of DE-52. A gradient of 0.075-0.5 M $(NH_4)_2SO_4$ in buffer C was run following a 10 column volume wash. Individual fractions were assayed for activity in the presence (broken line) or absence (tringles) of $5x10^{-7}$ M AMA.

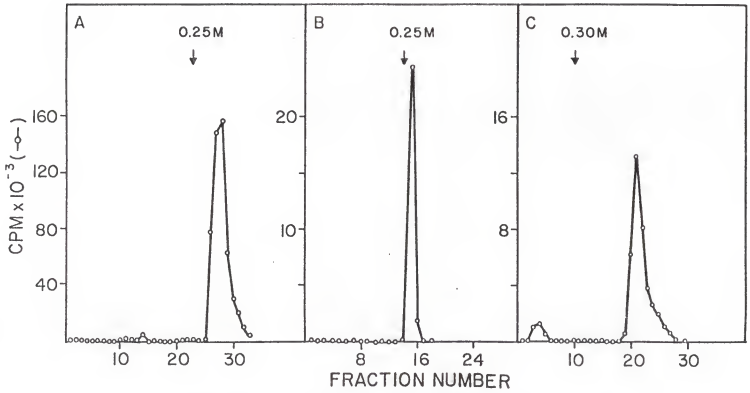


Figure III-3. Step-Elution Purification of Carrot RNA Polymerase II.
Panels A, B, and C profile the elution as activity (open circles) of RNA polymerase II from DE-52, phosphocellulose, and DNA-agarose, respectively. RNA polymerase II in individual fractions was assayed as described in the text.

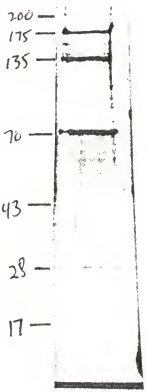
Additionally, by this step little if any detectable RNA polymerases I or III were present as indicated by complete sensitivity to $5x10^{-7}$ M α-amanitin.

Figure III-3 depicts the step elution of RNA polymerase II activities from DE-52, phosphocellulose, and DNA-agarose columns (panels A, B, and C). The appropriate volume for rinsing the columns prior to elution was determined in previous preparations lacking carrier protein; thus protein concentrations are not shown here. No loss of activity in flow through fractions is seen except for a small amount in the final step. Total recoveries of activity for each step of purification are listed in Table III-1.

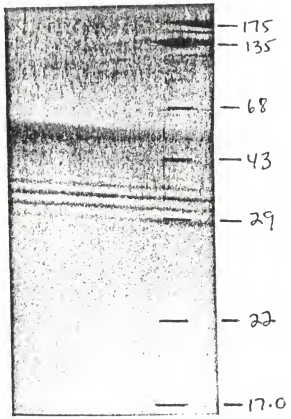
Subunit Composition of Carrot RNA Polymerase II

Figure III-4 compares the proteins comprising the most highly purified fraction (4) of RNA polymerase II. The gel in panel A demonstrates proteins expected for RNA polymerase II of apparent molecular weights of 175 (and 200) 135, 43, 28, and 17 kd as well as a protein of 70 kd. To corroborate subunits from this gel to those copurifying with genuine RNA polymerase II, enzyme was first subjected to electrophoresis under native conditions and then under denaturing conditions (panel B). In the latter case, it is apparent that all of these proteins associate with RNA polymerase II in addition to an expected subunit of 22 kd.

Figure III-4. Polyacrylamide Gel Electrophoresis of DNA-Agarose Purified RNA Polymerase II.
Peak fractions of the DNA-agarose column were subjected to polyacrylamide gel electrophoresis through a one-dimensional SDS gel (Panel A) or one-dimensional native(horizontal)/two-dimensional SDS gel and stained as described. Apparent molecular weights in kilodaltons are as shown. Protein bands below 135Kd which were poorly reproduced in the photograph were enhanced in ink. Broad bands between 27 Kd-35 Kd and 43 Kd-68Kd are considered artifactual.



A



B

Binding of ^{3}H-Amanitin to RNA Polymerase II

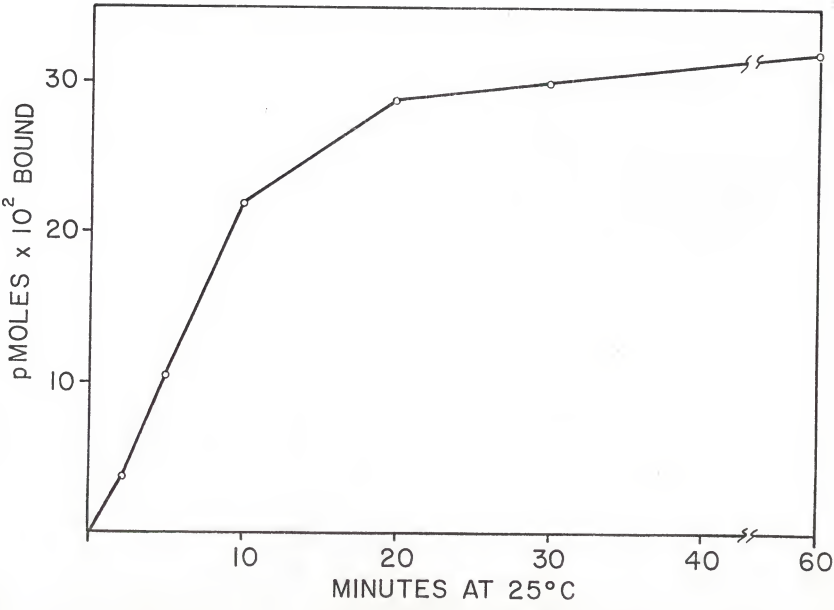
Figure III-5 describes the time requirement for complete association of ^{3}H-labeled amanitin with wheat germ RNA polymerase II. It is apparent that at least 30 minutes are required for equilibrium to be reached, with a $t_{1/2}$ = 7.5 minutes. For this reason, all subsequent *in vitro* assays with amanitin have adopted a preincubation with inhibitor prior to initiation of transcription.

The titration of RNA polymerase II with varied amounts of labeled amanitin and the subsequent linear transformation of these data to provide a Scatchard plot (Scatchard, 1949) is given in Figure III-6. Given the volume of assay and specific activity of labeled amanitin, a K_D of 1.5×10^{-11} M may be calculated. The comparison of this value to the kinetic estimation of K_I is given below.

Kinetic Estimation of K_I Values for Amatoxin Inhibition

The *in vitro* kinetic estimate of the K_I values for inhibition of carrot (panel A) and wheat germ (panel B) RNA polymerases II is presented in the Dixon plots of Figure III-7. Panel A shows an apparent K_I for α-amanitin of 4.5 nM for AMA or deAMA and 3.8 nM for meAMA when assayed with purified carrot RNA polymerase II. Wheat germ RNA polymerase II (panel B) appears only subtly different with an apparent K_I of 5.5 nM. Additionally, modifications on the amanitin indole ring appear to make little if any

Figure III-5. Time Requirement for Binding of ^{3}H-Amanitin to RNA Polymerase II. Purified wheat germ RNA polymerase II was incubated with labeled amanitin for the times indicated. Following the protocol in the text, individual tubes were collected on nitrocellulose disks and radioactivity determined.


pMOLES x 10^2 BOUND
30
20
10
10
20
30
40
60
MINUTES AT 25°C

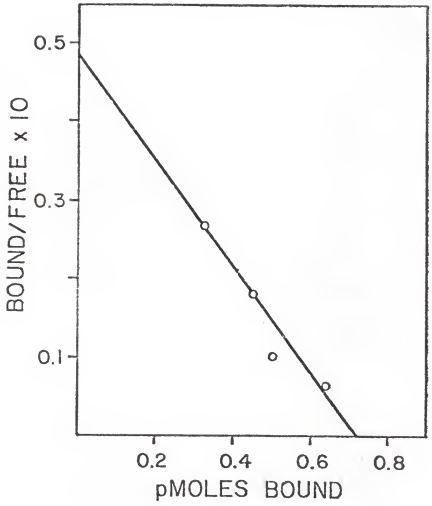
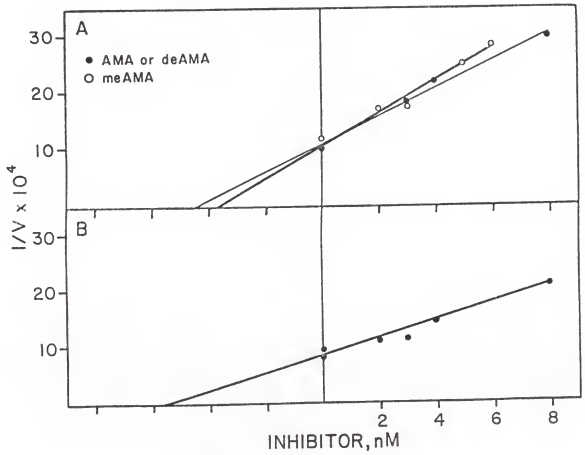


Figure III-6. Scatchard Plot of ^{3}H-Amanitin Binding to Plant RNA Polymerase II. Varying amounts of labeled amanitin were incubated for 30 minutes at 25C with a constant amount of wheat germ RNA polymerase II (0.25 µg). RNA polymerase-labeled amanitin counts were collected as described and radioactivity determined.

Figure III-7. Dixon Plots for Amatoxin Inhibition of Plant RNA Polymerase II. Purified carrot (panel A) or wheat germ (panel B) RNA polymerase II was assayed as described in the text with AMA or deAMA (closed circles) or meAMA (open circles). Apparent K_I values for wheat germ RNA polymerase II (AMA, 5.5 nM) or carrot RNA polymerase II (AMA or deAMA, 4.5 nM; meAMA, 3.8 nM) were derived graphically from intercepts on the inhibitor axes.


A
B
• AMA or deAMA
O meAMA
30
20
10
30
20
10
I/V x 10^4
2
4
6
8
INHIBITOR, nM

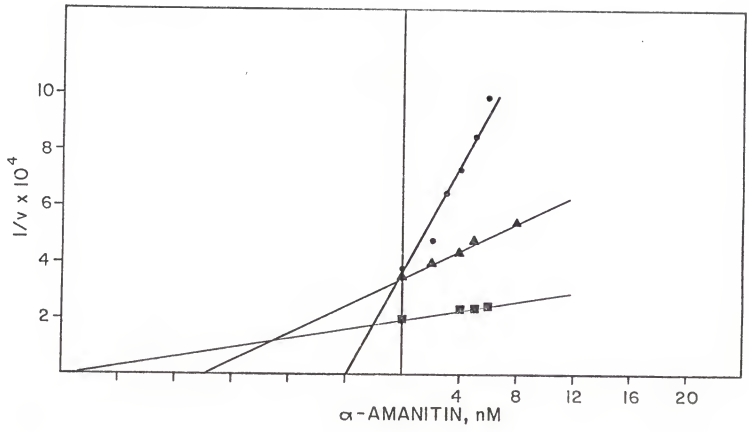
difference in the concentration of amatoxin needed for equivalent inhibition.

Figure III-8 demonstrates the genuine existence of the phenomenon relating apparent K_I and enzyme concentration. As shown, when enzyme concentration is varied from 28 to 55 units of activity (17 nM and 33 nM final enzyme concentration, respectively), the activity of the enzyme yields apparent K_I values of 14 and 23 nM. The lowest apparent K_I (4.5 nM) occurs at low enzyme concentration (1 unit or 0.6 nM enzyme), thus demonstrating that apparent K_I values generated at high enzyme concentrations can be quite varied.

Discussion

The purification of RNA polymerase II from carrot is generally similar to methods used for other plant polymerases II except for the inclusion of PVP to prevent inactivation of the enzyme by phenolic compounds produced by plants (Sekiya and Yamada, 1974). An important observation derived from the DE-52 gradient elution is that carrot (and wheat germ) RNA polymerases II tend to elute earlier than if Polymin P were omitted. Other workers (Engelke et al., 1983; Lerbs et al., 1983) have noted this anomolous behavior on DEAE-substituted celluloses and Sephadexes and a natural conclusion is that the cationic properties of residual Polymin P are the cause. A more reproducible elution may be seen if DEAE-Sepharose is used before DE-52. This resin binds polymerase II at an ionic strength (0.3 M) high enough

Figure III-8. Variation of Apparent K_I Values with Enzyme Concentration. Carrot RNA polymerase II was assayed in varying doses of α-amanitin; enzyme amounts were 1 unit (closed circles), 28 units (triangles), or 55 units (squares) for each of the three plots. Apparent K_I values (4 nM, 14 nM, 23 nM, respectively) were determined graphically from axis intercepts.


1/v x 10^4
10
8
6
4
2
4
8
12
16
20
α-AMANITIN, nM

to dissociate Polymin P from the polymerase with Polymin P appearing in the flow through fractions (unpublished observations).

The purification described here lending a 20% yield of RNA polymerase II in 2-3 days (approximately 100 µg RNA polymerase II per kilogram cells) is the second report of this enzyme from plant cell cultures. Link and Richter (1975) reported the isolation of parsley RNA polymerase II from suspension cell cultures. These authors found subunits of 200, 180, 140, 43, 26, 25, and 16 kd associated with an activity eluting from a DEAE-Sephadex column. Thus, carrot would appear to be more similar to parsley in this regard than to wheat germ RNA polymerase II (lower molecular weights of 40, 27, 25, 21, 20, 17.8, 17, 16.5, 16, and 14 kd) or soybean (subunits of 131, 42, 27, 22, 19, 17.6, 17, 16.2, 16.1, and 14). However, the complete profile of subunits associated with the plant class II transcriptional complexes will likely require gentler methods for purification, since subunits are known to be stripped from calf thymus RNA polymerase II during column chromatography (Robbins et al., 1984).

As mentioned earlier, several apparent K_I values for amanitin inhibition of plant polymerase II have been published, ranging from 50 to 170 nM. I have demonstrated that carrot and wheat germ enzymes are in fact much more sensitive than this with a lowest measurable K_I of 4.5 nM for these enzymes. Further, while it is apparent that such

variable K_I values already mentioned may be due to errors in amatoxin calibration, more likely reasons are incomplete binding of inhibitor during assay time and variable enzyme concentration used among reports. As shown in Figure III-8 when enzyme concentration is high, K_I values change almost linearly. There is precedence for such a relationship (Dixon and Webb, 1979) where tight binding inhibitors (K_I = nM) interact with enzymes other than RNA polymerase II. Although the K_I and K_D values derived from these experiments are not completely coincidental, the K_I value (4.5 nM) and K_D value (1.5×10^{-11} M) for the plant enzymes more closely resemble those values for animal cells (K_I = 2 nM, $K_D = 1.2 \times 10^{-11}$ M, Crerar et al., 1977) and further substantiates the claim of conservation of the amanitin binding site between these two systems.

The similarity between plant and animal RNA polymerase II interaction with amanitin would suggest an extreme conservation of the amanitin binding site across evolutionary boundaries. The significance of this may relate to the common features between the transcriptional apparatus of the two. Recently, Robbins et al. (1984) have shown that polyclonal antibody against Drosophila RNA polymerase II 220 and 140 kd subunits is also strongly cross reactive against the corresponding subunits in human, calf, chicken, and wheat germ enzymes. Additionally monoclonal antibody against calf thymus RNA polymerase II 220 kd subunit, that has been shown to inhibit binding to DNA, also binds

tenaciously to the large subunit of wheat germ enzyme. Collectively, this evidence suggests that basic functional requisites for transcription, i.e., template binding and polymerization, have been extremely conserved across the boundaries segregating these organisms, and further, that the transcription of specific genes by these enzymes may be governed by the smaller (< 40 kd) subunits.

SECTION IV

SENSITIVITY TOWARDS AND DEGRADATION OF AMATOXINS BY CARROT CELLS

Introduction

The selections of amanitin resistant lines of animal cells have shown the ease of selection of RNA polymerase II mutants in mammalian cells and suggests the feasibility of obtaining such lines in plants, (given the similar sensitivity of their RNA polymerases II to α-amanitin, see Section III). In this regard, since transcriptase mutants comprised the majority of such resistant lines, with perhaps one transport-deficient line recovered as well(Crerar and Pearson, 1977), one would expect the recovery of RNA polymerase II mutants in plants to be fairly straightforward. In fact, an attempt to obtain such mutants in tobacco failed, with calli initially showing resistance later becoming unstable, such that no RNA polymerase II mutants emerged (R. Grisebach, personal communication). While selections were underway (see Section V) in my experiments, a report appeared claiming the recovery of RNA polymerase II mutants (also in carrot, Vergera et al., 1982) through the use of α-amanitin as a selecting agent. These (2) mutants were recovered from a background of 24 resistant calli;

however, the data claiming altered polymerases II is unsubstantiated and possibly explained by levels of RNA polymerase I in those lines.

While it may be possible that RNA polymerase II mutants of plants can be obtained using α-amanitin with some plant species, the in vitro degradation of α-amanitin described below demonstrates that inhibitor loss can make this difficult, if not impossible. I have studied the responses of carrot cells to several derivatives of α-amanitin and will show that wild type cells possess the ability to degrade the parent α-amanitin, apparently through oxidation. Thus, since over one-half the α-amanitin will be shown to be lost in 5 days and since selections for plant cell mutants may require several months of exposure to an inhibitor, the level of α-amanitin critical for killing wild type cells may never be maintained. Fortunately, semisynthetic and naturally-occurring derivatives of α-amanitin do exist that are not inactivated, and the two used in this study are shown to more efficiently kill cells.

Materials and Methods

Reagents and Inhibitors

Glass-distilled deionized water was used for the preparation of all solutions, chromatography solvent mixtures, and plant media. Solvents used for high performance liquid chromatography (hplc) were hplc grade. Tritiated

thymidine (^{3}H-TdR, 500 μ Ci/ml, 20 Ci/mmole) was purchased from Schwartz Mann. Cycloheximide was purchased from Sigma and amatoxins were purified and characterized as described in Section III. Enzymes for protoplast production were obtained from Sigma (pectinase), Rohm and Haas (hemicellulase) and Calbiochem (cellulase).

Cell cultures and Protoplast Generation

Suspension cultures of Daucus carota Camden Hybrid were derived from axenic plants by culturing petiole sections on MS salts medium supplemented with 3% sucrose, 0.2% casamino acids, 200 μg/L kinetin and 100 μg/L 2,4-D (Ammirato, 1984). Cultures of the White Belgian cultivar, derived similarly, were obtained from David E. Evans of DNA Plant Technology Corp. (Cinnaminson, NJ). For convenience in media preparation, 2, 4-D and kinetin were prepared as the sodium and hydrochloride salts respectively by dissolving each in aqueous solutions containing one equivalent of NaOH or three equivalents HCl and lyophilizing the filtrates (0.2 μ GVWP filter). Callus cultures were maintained (weekly transfers) in the dark on medium supplemented with 1% agar while suspension cultures (3-4 days between transfer) were maintained on a gyrotary shaker (New Brunswick G-10, 150 rpm) at 25° C, 3000 lux and 12:12 hr.photoperiod.

Protoplasts were isolated from log phase suspension cultures by the procedure of Caboche (1980) using cellulysin (1%), pectinase (1%) and hemicellulase (0.5%) enzymes that

were individually desalted on Biogel P-6 (Evans and Bravo, 1983). Protoplasts generated after 4 h incubation with protoplasting enzymes were washed several times in W 0.6 macrosalts using low speed (100x g) centrifugation and resuspended in KM 8 P medium (Kao and Michayluk, 1975) at 3-6 x 10^5 cells/ml for inhibitor studies.

Labeled Thymidine Incorporation

To assess the relative cell responses to inhibitors, additions were made as follows using a protocol analogous to that of Cress et al. (1978). Suspension cultures were sieved to 300 µ with Nitex screen and collected in a 10 ml beaker containing a stir bar. Aliquots (90 µl) were removed from the stirred culture and transferred to a 10 x 75 mm test tube using 2 mm bore pipet tips. Following the addition of 10 µl of inhibitor (in water) or water, the tubes were placed in a capped humidified ointment jar. At 120 h, 10 µl 5-[^{3}H]-thymidine were added to each tube and incorporation allowed. At 24 h, the tubes were placed on ice and 1 ml of ice-cold 10% TCA was added. Tube contents with TCA rinsings were quantitatively collected on GF/C (Whatman, 2.4 cm) filters, were washed, dried and counted as described by Preston et al. (1975). Percent survival is expressed as the ratio (x 100) of cpm obtained for a particular inhibitor dose to control cpm obtained with water only.

The protocol for protoplast response was similar. One hundred µl of protoplasts were incubated in microtiter

plates with 10 µl inhibitor for 72 h under low light (300 lux). Ten µl of ^{3}H-TdR were added; after allowing 24 h incorporation the amount of TCA-insoluble radioactivity was determined as for suspension cells.

Vital Dye Staining of Protoplasted Cells

To corroborate thymidine incorporation as a measure of cell viability a comparison was made as follows. A 24 h culture of suspension cells (100 µl sieved to 300 µ) incubated for 48 h with 10 µl of inhibitor or water. At 48 h 10 µl of either ^{3}H TdR or water (for vital staining of cells) was added and incubated an additional 24 h. On the third day cells were either harvested for the assessment of radioactivity incorporation (as above), or converted to protoplasts with an equal volume of protoplasting enzyme solution. Viable cells were counted in duplicate immediately after the addition of an equal volume of 0.4% Evan's Blue in T_0 + sucrose (Taylor and West, 1980).

High Performance Liquid Chromatography

Reverse phase hplc was performed isocratically with 19% acetonitrile as described for virotoxins (Little and Preston, 1984) or by using a Waters RCSS 2 module packed with µbondapak C18 and a flow rate of 2 ml/min. A Waters 721 System Controller, 730 Data Module and WISP automated injector provided microprocessor pump (M6000 A) control, data handling and sample injection. A Gilson Holochrome

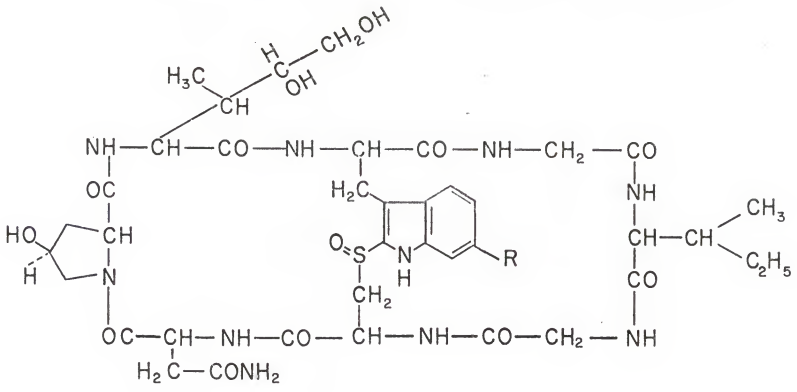
variable wavelength detector fitted with a 1.00 cm cuvette was used for monitoring elution.

Amatoxin Inactivation

The loss of amatoxins in supernates was measured as follows. Ten µl of inhibitor or water was added to 90 µl cells and incubated in duplicate in microtiter wells for 5 days on a shaker as described above. Cells were allowed to settle to the bottom of the wells and 40 µl of supernatant were removed, diluted to 200 µl with water and stored in 0.3 ml low headspace vials (Wheaton Micro Pak) at -20° C until analysis. Toxins in the supernatants were resolved by automated HPLC and recoveries established by electronic integration of peak areas relative to control wells (lacking cells) under identical conditions of exposure.

ß-Elimination

Relative rates of ß-elimination for amatoxin derivatives were measured using a protocol similar to that of Faulstich et al. (1980). Aqueous solutions (1 ml) of the inhibitors were prepared to give a final concentration of 20 µM. Ten µl of 1 M NaOH were added to each reaction mixture at zero time and subsequently neutralized at the indicated times (Figure IV-1) with 10 µl 1 M HCl. At 3 hours the absorbances of all reactions were measured at either 304 nm (for AMA and meAMA) or 287 nm (deAMA).



		R
α- Amanitin		OH
6'- O-methyl-α amanitin	meAMA	OCH_3
6'- deoxy-α-amanitin	deAMA	H

Figure IV-1. Structures of Amatoxin Derivatives.
This figure depicts the differences and common features in the chemical structures of amatoxins used in these studies.

Results

^{3}H-TdR as a Measure of Cell Viability

Table IV-1 compares the measure of cell viability using both labeled thymidine incorporation and vital dye staining of cells exposed to various amanitin derivatives and cycloheximide (CHX). As can be seen, 2 day exposures to the inhibitors are effective in killing cells when measured in either manner. A reasonable correlation is thus drawn between labeled thymidine incorporation and a given inhibitor dose, with the possible exception of meAMA. In the latter case, survival appears greater with the vital stain than with the incorporation of ^{3}H-TdR. It is probable this difference is not real since there is no *a priori* reason to believe meAMA, to the exclusion of the other amatoxins, would have such a differential effect. Thus, in subsequent experiments ^{3}H-TdR incorporation will be considered to reflect *bona fide* survival capacity of cells.

Figure IV-2 demonstrates that ^{3}H-TdR incorporation can be followed for nearly one week in newly transferred cells using the protocol above. Further, this incorporation may be followed as a 24 h pulse or continuously; in the latter instance counts are reflected as additive ordinates from 24 h pulse cpm. This system would thus appear to provide a reasonable and rapid approach to quantitating cellular responses to inhibitors over many days of incubation.

Table IV-1. Comparison of ^{3}H-TDR Incorporation with Vital Dye Staining to Measure Cell Viability.

^{3}H-TdR			Cell Counts	
CPM	% Control	Inhibitor, M	Cells/ml	% Control
24853	100	None	1.5×10^{6}	100
6412	26	AMA, 4.5×10^{-4}	3.6×10^{5}	24
4024	16	meAMA, 3.1×10^{-4}	5.6×10^{5}	37
4351	18	deAMA, 1.7×10^{-4}	3.2×10^{5}	21
4010	16	CHX, 9×10^{-6}	3.6×10^{5}	24

Suspension cells were incubated 48 h with the inhibitors at final concentrations listed. For thymidine incorporation, cells were pulsed for 24 h with ^{3}H-TdR and processed. Vital dye staining of protoplasted cells was performed on the third day of inhibitor incubation and viable cells determined as described in the text.

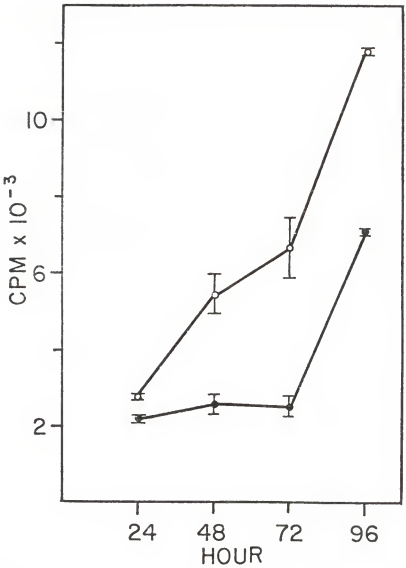


Figure IV-2. Incorporation of Labeled Thymidine by Carrot Suspension Cells.
Suspension cells were labled using a 24 h pulse (closed circles) or by allowing continuous incorporation (open circles) of labeled thymidine into DNA. At the indicated times, cells were harvested and tritium incorporation determined as described in the text.

Survival of Protoplasts and Suspension Cells Exposed to Amatoxins

Figure IV-3 compares the relative sensitivities of protoplasts and suspension cells after 4 days exposure to AMA and meAMA. An examination of doses giving 50% survival indicates cells to be approximately tenfold more sensitive than protoplasts to meAMA or AMA. At the same time a severalfold difference in sensitivities to AMA and meAMA is apparent in either case.

The response of suspension cells only to these inhibitors (and deAMA) is given in Figure IV-4. This response, measured at 5 days, shows a striking difference in the response given by AMA versus that for meAMA or deAMA. A comparison of the 50% survival doses for these 3 derivatives along with K_I values (from Section III) for their target protein -RNA polymerase II- is listed in Table IV-2. Clearly the derivative (AMA) possessing a 6'-hydroxyl group on the peptide indole ring is least effective for cell inhibition relative to deAMA and meAMA (see Figure IV-1).

Recoveries of Amatoxins from Culture Supernatants

The profiles of Figure IV-5 present the recoveries, as evaluated by hplc, of AMA (A,B) and meAMA (C,D) from supernatants of suspension cells cultured for 5 days. The presence of cells (D,D) (versus wells with no cells, A and C) reduces the recovery of AMA but not meAMA. Corroboration of this fact by assaying supernatants with calf thymus RNA

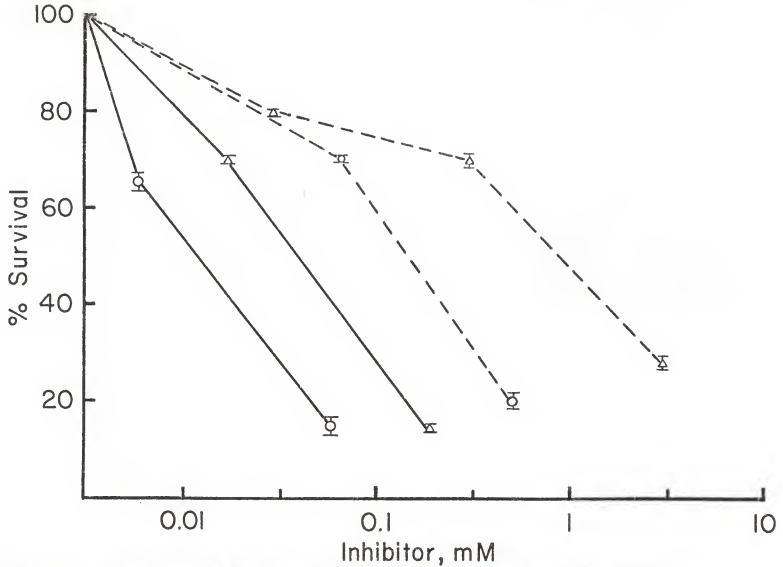


Figure IV-3. Response of Suspension Cells and Protoplasts to Amatoxins. Suspension cells (——) or protoplasts (---) were exposed for 72 h to either meAMA (closed circles) or AMA (triangles). Following a 24 h pulse with ^{3}H-thymidine, cells were collected by filtration and survival determined as described in the text.

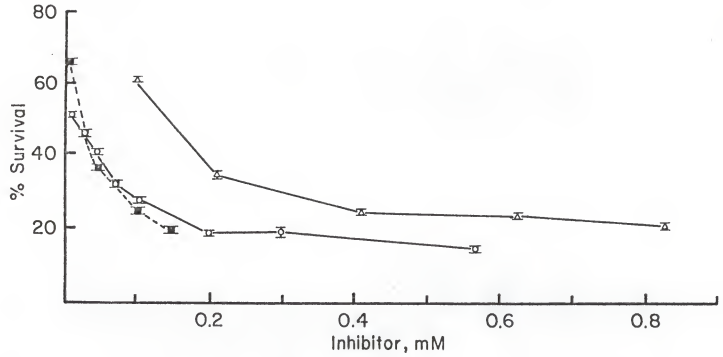


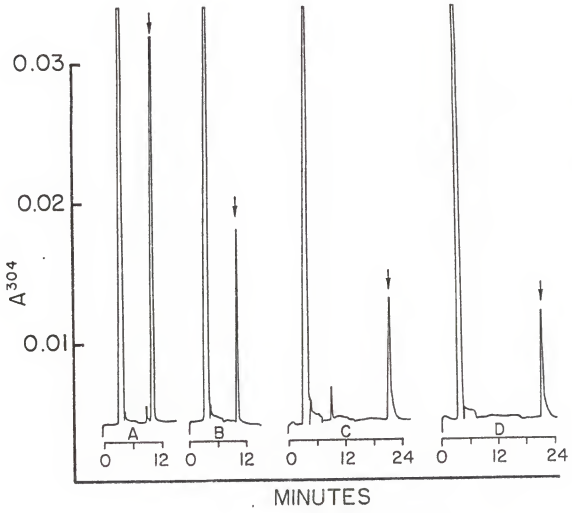
Figure IV-4. Comparsion of Responses by Suspension Cells to Amatoxins. Cells were incubated for 96 h with either AMA (triangles), meAMA (open circles), or deAMA (squares) and pulse labled for 24 h with labeled thymidine. Survival is determined relative to cells given no inhibitor for the first 96 h.

Table IV-2. Comparative Efficiencies of Whole Cell and RNA Polymerase II Inhibition by Amatoxins.

Toxin	ID_{50}, μM	K_I, nM	Efficiency (AMA = 1)
AMA	140	4.5	1
deAMA	25	4.5	5.6
meAMA	17	3.7	6.8

Doses giving 50% inhibition of cell survival (ID_{50}) or 50% inhibition of RNA polymerase II (K_I) are compared. The efficiency of killing is the ratio of K_I to ID_{50}, normalized for AMA.

Figure IV-5. Amatoxin Loss in Cell Culture Supernatants.
Cells (B,D) or media (A,C) were incubated 5 da with either AMA (A,B) or meAMA (C,D) and supernatants evaluated by hplc. Arrows indicate the elution positions of inhibitors.


0.03
0.02
0.01
A304
A
B
C
D
0
12
0
12
0
12
24
0
12
24
MINUTES

fact by assaying supernatants with calf thymus RNA polymerase II (Preston et al., 1975) suggested losses of AMA to be 50% to 80% while the meAMA was quantitatively recovered (not shown). Quantification of recovery of these two amatoxins along with that for deAMA is given in Table IV-3. This demonstrates the inactivation of only AMA cccurs and that this loss is apparent even at low doses, while the two derivatives lacking the 6'-hydroxyindole moiety are completely recovered. Protoplasts exposed in a similar manner also destroy AMA, while no loss of meAMA is seen (Table IV-4). Additionally, and significant relative to mutant selection, no metabolic conversion of deAMA or meAMA to AMA (which would subsequently be degraded) by 6'-hydroxylation or 6'-O-demethylation is apparent.

The tubes presented in Figure IV-6 reflect changes in the supernatants of cells exposed for 5 days to AMA, meAMA, or CHX. The doses of inhibitor were intentionally excessive over that needed for killing to demonstrate the yellow supernatant unique to cells exposed to AMA. That this is not due to nonspecific synthesis of phenolics, generated in response to a trauma, is shown by the absence of this yellow supernate in the CHX tube.

ß-Elimination

To ask whether the metabolic loss of AMA was due to a difference in leaving group potential of the sulfoxyindole moiety of this peptide, the rate of ß-elimination for AMA

Table IV-3. Inhibitor Recoveries by hplc of AMA, deAMA, and meAMA from Cell Culture Supernatants.

Inhibitor, µM		Average % Recovery
AMA	50	55 ± 9
AMA	100	59 ± 3
AMA	200	54 ± 1
AMA	400	58 ± 6
meAMA	50	96 ± 6
meAMA	100	100 ± 3
meAMA	200	105 ± 2
meAMA	400	115 ± 6
deAMA	50	98
deAMA	100	98 ± 1
deAMA	200	101 ± 7

Inhibitor levels were determined in cell culture supernatants after 5 da incubation with cells. Supernatants were assayed by reverse phase hplc and peak area quantitation used to provide average percent recoveries of duplicate samples.

Table IV-4. Inhibitor Recoveries by hplc of AMA and me AMA from Protoplast Supernatants.

Inhibitor, µM		Average % Recovery
AMA,	100	80
meAMA,	100	97

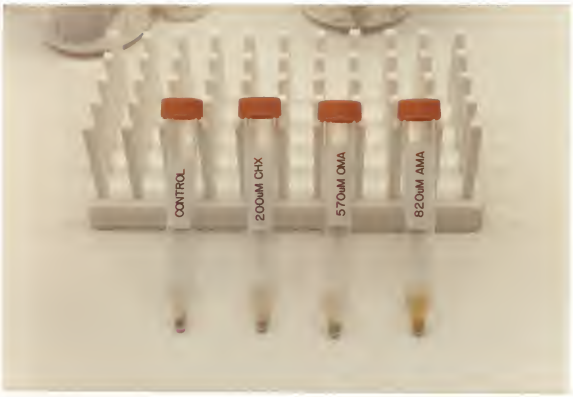


Figure IV-6. Visible Signs of AMA Degradation. Tubes containg α-amanitin (AMA), 6'-O-methyl-αamanitin (OMA in this photo), or cyclohexi-mide (CHX) at the concentrations listed were incubated 96 h with suspension cultures of carrot. Note the yellow supernatant in the tube containing AMA.

was compared to the other derivatives. Figure IV-7 shows this elimination (see Figure IV-8 for reaction mechanism) as carried out at pH 12. Although the final absorbances of the rearranged product derived from each of these homologs are different, the relative rate of elimination is the same for all three. Thus, a common $t_{1/2}$ of 42 minutes suggests no intrinsic differences in the rates of β-elimination for these compounds; this mechanism cannot then explain the selective loss of AMA from cell culture supernates.

Discussion

The survival response measured here for amanitin is similar to that observed for parsley callus (Seitz and Seitz, 1971), Kalanchoe leaves (Beiderbeck, 1972), tomato protoplasts (Rackwitz et al., 1981) and most recently carrot (Vergera et al., 1982); however, as shown in Figure 3, cells appear approximately tenfold more sensitive than protoplasts (also seen in tobacco, J.F. Preston, unpublished). One likely basis for this discrepancy is that of differential uptake between walled and protoplasted cells. It would appear that simple diffusion alone is not the only factor governing entry into these cells but rather a factor(s), perhaps linked to cell wall components, that was rendered nonfunctional by treatment with protoplasting enzymes. Additionally, the increased lipophilicity of meAMA and the inactivation of AMA (infra vide) explain the differential responses of protoplasts to these two derivatives.

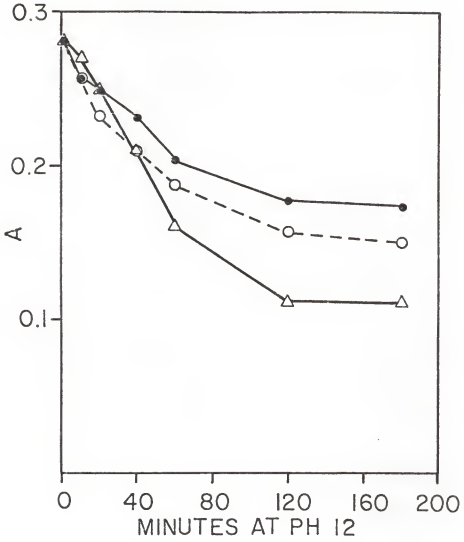


Figure IV-7. Relative Rates of β-Elimination of Amatoxins. Aqueous solutions of either AMA (triangles), meAMA (open circles), or deAMA (closed circles) were incubated at pH 12 for the indicated times, neutralized, and absorbances at either 304 nm (AMA, meAMA) or 287 nm (deAMA) determined. In each derivative one-half of the molecules have undergone β-elimination by 42 min.

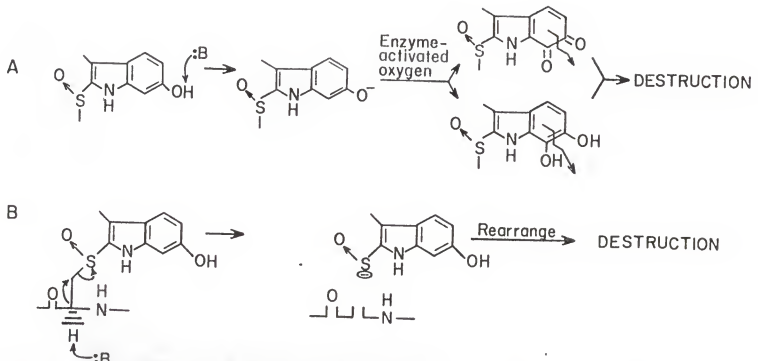


Figure IV-8. Mechanisms for Amatoxin Inactivation.
Scheme 8A represents inactivation of the indole ring of AMA through initial ionization of the 6'hydroxyl to a phenolate. The acquisition of an oxygen atom to provide a 6', 7'-dione or 6', 7'-diol provides a hypothetically unstable ring. Scheme 8B presents general-base catalyzed β-elimination which might be catalyzed by NH_2 or other protein nucleophiles. This mechanism would cleave the bicyclic ring yielding dehydroalanine in place of cysteine and a mesomeric sulfoxyindole which is hypothesized to rearrange.

Data from Figure 4 relating the 50% inhibition doses for suspension cells given by AMA, meAMA, and deAMA are tabulated (Table IV-2) for comparison with the K_I for RNA polymerase II for these same derivatives. It is clear that meAMA and deAMA are better inhibitors of whole cells than AMA itself by five to sevenfold. In part this may be explained by the loss of approximately one-half the AMA to inactivation by the cells, with less AMA then available for inhibition. A second reason AMA is least effective is probably the lesser lipophilicity of this derivative compared to deAMA and meAMA, since the latter two elute from a reverse phase hplc column after AMA. Evidence for this is the increased toxicity toward white mice and Drosophila embryos seen with derivatives of AMA modified to be more lipophilic (Faulstich et al., 1981). The increased sensitivity of cells to meAMA vs AMA has also been noted in tobacco (J.F. Preston, unpublished).

Analyses by hplc showing the selective inactivation of AMA are given in Tables IV-3,4 and Figure IV-5. This inactivation indicates one basis for the refractory nature of cells to AMA versus for deAMA and meAMA. Such inactivation, which was seen for both cultivars of carrot, could be explained in two ways. In Figure IV-8a one of the two possible mechanisms of destruction is suggested to be an oxidation of the hydroxyindole moiety. This oxidation is hypothesized to be first brought about by phenolate formation at the 6'-carbon. Subsequent formation of a 6,

7-dioxygenated ring, which would be expected to rearrange and polymerize or destruct, could be promoted by an enzyme-activated oxygen species. Evidence on behalf of this mechanism is 1) Proton Overhauser and X-ray studies showing that the indole ring is disposed away from the peptide backbone and hence probably accessible to enzymes (Wieland et al., 1983), 2) the known destruction of AMA mediated by lactoperoxidase (Morris et al., 1979), $NaIO_4$ (Wieland, 1983), and nontoxic mushrooms (Romeo and Preston, 1984), 3) the yellowish appearance of culture supernatants (in Figure 6 and also noted with tobacco protoplasts, J.F. Preston, unpublished) exposed to AMA, suggesting an altered conjugation system for the indole ring and 5) known substrates for polyphenoloxidases (Mayer and Hayer, 1979). Although mechanism 8b would also destroy the ring and yield a product noninhibitory to RNA polymerase II, the rates of β-elimination among the derivatives are equal (Figure 7). Thus some form of mechanism 8b is more likely.

The major importance of the selective degradation of AMA relates to the acquisition of transcriptional mutants and plant cell lines resistant to other inhibitors as well. The fact that the RNA polymerase II locus is present in only two copies in diploid animal cells and insects (Greenleaf et al., 1980; Crerar et al., 1977) makes it an attractive gene to selectively target for mutation. Vergara et al. (1982) have recently claimed to have isolated RNA polymerase II mutants from carrot using α-amanitin (50 μg/ml) as a

selecting agent; however, scrutinization of their data shows such a claim is unsubstantiated. Attempts to isolate cycloheximide ribosomal mutants in plants have been totally unsuccessful, despite the fact that such mutants are easily obtained from other eukaryotes (Gresshoff, 1979; Maliga et al., 1976; Fried and Warner, 1982); only recently have wild type cells of carrot been demonstrated to degrade cycloheximide (Sung, 1981). Thus it is clear that inhibitor degradation is not unique to α-amanitin; the assessment of inactivation of inhibitors should then be a logical first step prior to selecting resistant lines if detoxifying mutants are not sought. In the case of amanitin degradation, a survey of oxidase levels in candidate species may be desirable.

SECTION V

SELECTION AND CHARACTERIZATION OF CELL LINES RESISTANT TO METHYLAMANITIN

Introduction

Although cells and organisms possessing altered RNA polymerases II through resistance to α-amanitin have been isolated in Drosophila, animal cells, and Caenorhabditis worms, there have been no cogent reports of such mutants within the plant kingdom, inclusive of algae and higher plants (see Section I). The availability of such resistant lines and the demonstration of altered RNA polymerases II associated therewith would provide an extremely useful tool to study transcription in such organisms. The well-documented aberrations in developmental phenotypes associated with an altered RNA polymerase II of Drosophila (Mortin and LeFevre; 1981, Greenleaf et al., 1980) and rat myoblast cells (Crerar et al., 1983) would argue that such a well-defined mutation in a plant RNA polymerase II would provide a powerful and discrete means of probing the population of genes that are activated during plant development. Given the ready potential for regeneration from tissue culture in a number of species (Ammirato, 1983) such information would

doubtless contribute to the understanding of mechanisms governing this fascinating process as well.

While the experiments below were in progress, Vergera et al. (1982) reported the isolation of α-amanitin resistant lines of carrot using AMA concentrations of 50 µg/ml (50% of this is degraded in 5 days or less, see Section IV). These authors further suggested they had isolated two RNA polymerase II mutants, even though A) no RNA polymerase II was ever purified and assayed from these lines and B) no labeled AMA binding studies were done on these putative resistant enzymes. In fact, the data claiming resistant RNA polymerases II could easily be explained by backgrounds of RNA polymerase I in these lines. This is most clearly the case for data from their single AMA induction experiment, in which over a 70% loss of activity is apparent, and that which remains is clearly RNA polymerase I. Thus, while these authors may have isolated RNA polymerase II mutants there is no convincing evidence from this paper that such exist.

The experiments below describe the selection of cell lines of carrot which are resistant to meAMA. The choice of this selecting agent provides a derivative (see Section IV) of AMA that is both resistant to oxidative degradation -known to occur with AMA even in wild type cells- and apparently more readily taken up by cells. This should theoretically reduce the background seen with only AMA. These resistant lines are shown to be unable to degrade

meAMA. Additional experiments will examine the RNA polymerases in these lines.

Materials and Methods

Reagents and Solutions

Glass distilled and deionized water was used for the preparation of all culture media and buffers. Methanesulfonic acid ethyl ester (EMS) was obtained from Sigma Chemical and was filtered (0.2 µGVWP, Millipore) before use. Methylated α-amanitin (meAMA, prepared as described in Section II) stocks were prepared in water and filtered as above. Solvents for chromatography were hplc grade. Tritiated thymidine (^{3}H-TdR 500 µCi/ml, 20 Ci/mmol) was obtained from Schwarz Mann.

Cell Lines and Culture

Suspension cell cultures of D. carota White Belgian were obtained from David A. Evans (DNA Plant Technology Corp., Cinnaminson, N.J.) and maintained as described in Section III.

Response of Cells to Mutagenesis

A 24 h culture of suspension cells sieved (Nitex) to 300 µ or less was divided into 15 aliquots of 1 ml each. To each 15 ml conical centrifuge tube containing the 1 ml aliquots, 1 µl to 10 µl EMS was added and the suspensions

were incubated 2 h with shaking. At the end of 2 h, 10 ml 0.1 MSC was added and the suspension was centrifuged for 10 min at 1,000 x g. This washing is repeated and cells resuspended in 1 ml 0.1 MSC. Duplicates of 100 µl from each dose were delivered into sterile 10 x 75 mm test tubes along with 10 µl ^{3}H-TdR. Incorporation was permitted for 24 h, at which time TCA addition and filtration (as in Section III) were performed. Percent survival is expressed (cpm x 100) relative to cells initially given no EMS, but washed as for cells given EMS.

Selection and Characterization of Cell Lines Resistant to meAMA

The selection of cell lines resistant to meAMA was carried out as follows. A log phase culture of suspension cells was filtered through Nitex to provide clumps and single cells of 1 mm or less. Collected cells were pelleted by centrifugation (as above), resuspended in 250 ml 0.1 MSC, and counted by protoplasting 1 ml of the culture according to the methods in Section III. Approximately 175 g or 2.4×10^{8} cells were treated for 2 h with EMS at a final concentration of 0.3%. Following 4 washes with medium as described above, cells were resuspended in 200 ml fresh 0.1 MSC medium. The cells were grown up 4 days, washed twice with quantitative transfer, and resuspended in 200 ml 0.1 MSC for plating.

Petri dishes (16x150 mm) containing 20 ml 2% agar and meAMA (sufficient to provide 1% agar and 20 µM meAMA final)

were readied. Twenty ml of cells were then rapidly mixed into the dishes and stirred with a sterile glass rod for several minutes to prevent cell clumps from settling before the mixture congealed. Dishes were sealed with parafilm and placed in the dark.

An examination of the plates at 6 weeks revealed 5 calli of 3 mm size, which were transferred onto 1 ml dishes of 0.1 MSC in agar. These calli were initially tolerant of meAMA at 10 μM doses (not shown) but this tolerance was quickly lost.

After 3 months of incubation the original plates were reexamined. By this time approximately 10 calli of 0.5 cm in diameter had formed, and these were transferred to medium lacking meAMA. Subsequent challenge with 20 uM meAMA yielded 2 calli, B6 and B10. These lines which have remained resistant for over one year in the absence of meAMA, were cloned as callus from 20 μM meAMA. Relative responses of these lines were assessed by determining callus fresh weights after 2 weeks on meAMA.

Stability of meAMA in Cultures of meAMA-Resistant Lines

Cultures of lines B6 and B10 were incubated with 50 μM meAMA for 5 days. Supernatants were evaluated by hplc for recovery of meAMA as described in Section IV, except that absorbance of eluates at 212 nm was monitored.

Evaluation of RNA Polymerase Activities in Wild Type and Resistant Lines

RNA polymerase II activities were extracted from suspension cells as described in Section IV, and carried through the ammonium sulfate step (Fr. 2). Assays with AMA were as described in Section IV. Cells which were grown in meAMA were washed 5-6 times with 10 volumes each of 0.1 MSC prior to harvest and extraction.

Results

Response to Mutagenesis

Figure V-1 relates the survival of suspension cells to EMS. A dose of approximately 0.3% yields 50% survival of the culture, a value similar to that (0.35%) seen with soybean (Sung, 1976), when cell density is used to monitor survival. This dose was chosen for mutagenesis in the selection of meAMA resistant lines.

Characterization of meAMA-Resistant Lines

The response of wild type and lines B6 and B10 to meAMA is shown in Figure V-2. A 50% inhibition of growth of wild type callus can be seen at 2 μM meAMA whereas 14 μM and 16 μM doses are needed to effect the same in lines B10 and B6 respectively. A visual comparison of this response is shown in Figure V-3.

Table V-1 describes the recovery of meAMA from supernatants of lines B6 and B10 cultured for 5 days with

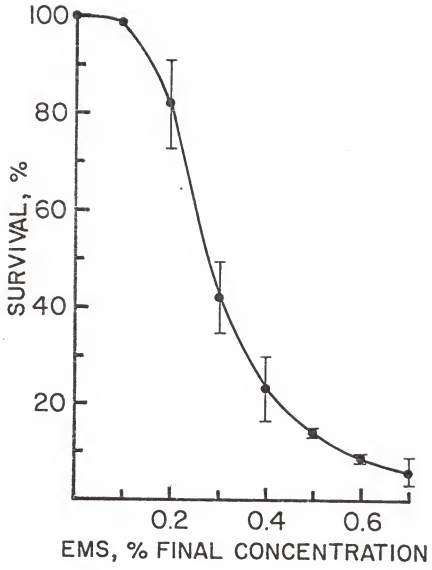


Figure V-1. Response of Carrot Suspension Cells to Mutagenesis.
Cells were exposed to EMS to provide the final concentration indicated. Survival was measured following a 24 h exposure to ^{3}H-TdR.

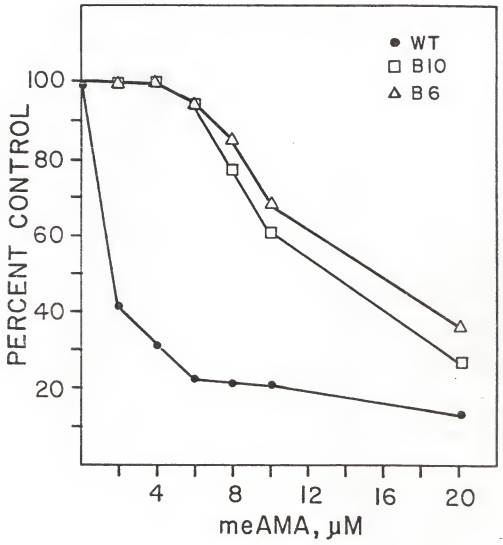


Figure V-2. Response of Resistant and Wild Type Cell Lines to meAMA.
Callus of wild type (solid circles) and lines B6 (triangles) and B10 (squares) were plated onto agar media containing meAMA at the indicated doses. Fresh weight of calli were determined to assess survival.

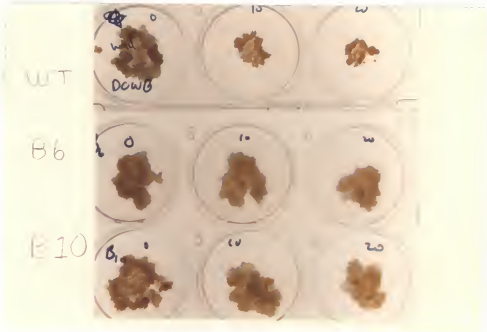


Figure V-3. Growth of Wild Type and Resistant Cell Lines on meAMA.
Callus of wild type and the meAMA-resistant lines B6 and B10 was grown on solid medium containing meAMA at the indicated doses (μM) for 2 weeks.

Table V-1. Recoveries of meAMA From Cell Cultures by Hplc.

Line	% Recovery
B6	104 ± 2
B10	96 ± 2

50 μM meAMA. In each case, the recovery is quantitative. This indicates these lines are not resistant as a result of being able to degrade the selecting agent.

RNA polymerase activities were examined in wild type and resistant cells under conditions in which 5 day growth occurred with or without 10 μM meAMA. The activities present in Fractions 1 (approximately 50% RNA polymerase I and 48% polymerase II) and 2 (comprised of 90-95% RNA polymerase II and a small amount of RNA polymerase I) under both conditions are given in Table V-2. From this table it is clear that wild type cells lose over 90% of their total RNA polymerase activities when grown with meAMA under conditions used to induce resistant RNA polymerase II in the resistant lines. These lines do not experience this loss (except for the loss of RNA polymerase II activities, infra vide) and appear to have roughly equivalent levels of activities, including a slightly higher background of RNA polymerase I. The titration of the Fraction 1 activities (Figure V-4) suggests this to be RNA polymerase I and

Table V-2. RNA Polymerase Activities in Wild Type and meAMA Resistant Lines.

Cell Line	meAMA Induced(+/-)	Fr. 1[a] cpm/g Tissue	Fr. 2[b] cpm/g Tissue
Wild type	-	3.3×10^4	1.7×10^4
	+	3.0×10^3	not assayed
B6	-	4.0×10^4	1.5×10^4
	+	2.1×10^4	2.3×10^3
B10	-	4.6×10^4	1.2×10^4
	+	3.3×10^4	2.4×10^3

a. Protein concentrations were 1.1 to 1.2 mg/ml.

b. Protein concentrations not determined due to interference by residual Polymin P.

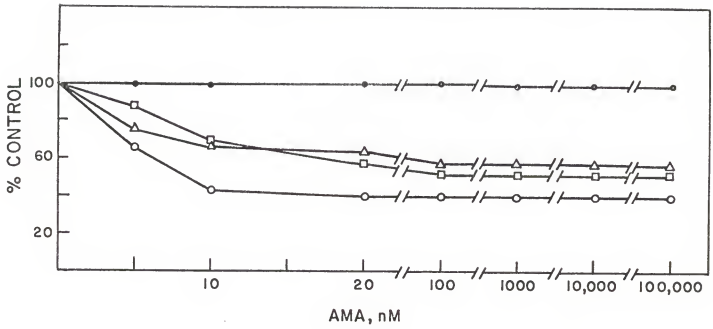


Figure V-4. RNA Polymerase Activities from Wild Type and Resistant Cell Lines. RNA polymerase activities present in crude lysates (Fr. 1) of wild type (open circles), B6 (squares), and B10 (triangles) cell lines grown in the absence of meAMA are titrated with the indicated doses of AMA. Also shown is Fr. 1 activity from lines grown in 10 μM meAMA for 5 days (closed circles).

establishes a similar identity for the activity elicited by meAMA induction in the resistant lines.

RNA polymerase II activities from the second fraction (polymin P eluate) were resolved from RNA polymerase I and residual polymin P by purification on DEAE Sepharose. Figure V-5 depicts the response of enzyme thus obtained to varying concentrations of AMA. The monophasic nature of the inhibition curves indicates no detectable RNA polymerase II with altered AMA sensitivity.

RNA polymerase II was purified through DEAE-Sepharose as described in the text. Aliquots from the main peak derived from wild type (closed circles) and B6 or B10 (triangles) cells was assayed with AMA at the concentrations indicated.

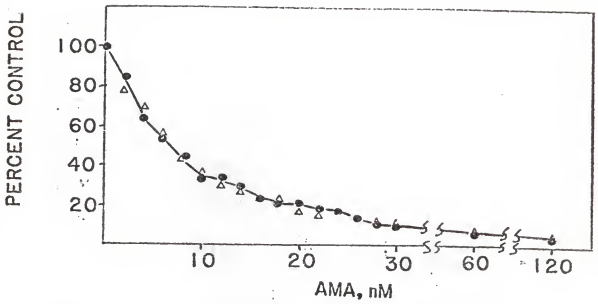


Figure V-5. Partially Purified RNA Polymerase II from Wild Type and Resistant Lines.

Discussion

The selection of these resistant lines conforms to methods generally used for selecting resistant plant cell mutants (Flick, 1983). In this regard, the adoption of a mutagen dose convenient to estimate survival (and therefore mutation frequencies), the sieving cell clumps to provide visual calibration in discriminating true resistant growth, and long incubation times are typical. In addition, cloning lines from the inhibitor and observing that resistance has been maintained for 1.5 yr in the absence of inhibitor, provides assurance that these are pure lines with stable resistance. Thus, in a single step 2 lines, each 7-8 fold resistant to meAMA over the wild type, have been isolated;

a calculated frequency of appearance of 3-7 x 10^{-7} (Flick, 1983). These lines have lost the capacity to regenerate plants as have the wild type.

The characterization of these lines is complete. It is clear that neither is capable of degrading the selecting agent, showing that the use of this agent is superior to α-amanitin, and that its use does apparently not select for O-demethylase activities which might convert the meAMA to AMA, with consequential degradation. Since the indole ring of AMA is known to be disposed away from the peptide backbone, an early supposition was that this epitope might mimmick that of IAA, and hence the response of these lines toward auxin might be different; however both behaved similar to the parental line in that 1-2 μM auxin caused 50% inhibition of growth (not shown). That these mutants are deficient in transport is a possibility, however no direct demonstration of this phenotype had been made in AMA resistant animal cells (Crerar and Pearson, 1977).

The assessment as to why these lines are meAMA resistant was undertaken using an analysis of RNA polymerase activities in cells grown in the presence and absence of meAMA. These data show that the lines grown in the absence of inhibitor appear to have a higher background of RNA polymerase I which slightly complicates analysis of a potential heterozygous mutation. Cells grown in the inhibitor demonstrate their refractory nature toward the drug (relative to wild type) under these conditions as well as a

loss of RNA polymerase II. Since the cells are clearly viable during this induction and one should recover at least wild type activity, it is probable that the loss of RNA polymerase II activity is attributable to trace amounts of amanitin present following washing; Crerar and Pearson (1977) were unable to demonstrate clear amanitin resistance in AMA^R mutants, that were induced for the resistant activity, without first eliminating traces of the inhibitor by mass action from the enzyme complex. However, the examination of RNA polymerase II activities suggests that if an alteration exists in this enzyme from the resistant lines, then such alteration is kinetically undetectable.

An interesting feature of the resistant lines is that RNA polymerase I levels appear 20 to 40% higher than that seen in wild type lines. This may also be the case with lines isolated by Vergera et al. (1982). Although a prejudice based upon animal cells resistant to AMA would bias the expectation of the exclusive appearance of RNA polymerase II mutants, it is possible other mechanisms might confer resistance. Such mechanisms, such as a generalized stress response, may require a mobilized array of ribosomes, thus lending a higher RNA polymerase I level. The validity of this and other hypotheses awaits the selection of meAMA resistant lines from other plant species.

SECTION VI

DEVELOPMENT OF AFFINITY SYSTEMS FOR RNA POLYMERASES II AND III AND THEIR TRANSCRIPTIONAL COMPLEXES

Introduction

The search for a precise determination of the proteins comprising enzyme systems that confer transcriptional fidelity, and indeed that of the core complex itself, has classically been attempted through exhaustive purification to constant specific activity of the RNA polymerases (see Section I). In the case of plant RNA polymerases II this has yielded a series of discrete polypeptides on SDS gels that migrate collectively on the native gels, but, like the animal enzymes, these preparations are unable to initiate specific gene transcription (Guilfoyle, 1981). In addition, it is difficult to prove whether or not comigrating peptides are part of the bona fide transcriptional machinery or merely copurifying contaminants. While there is consensus in the subunit composition of many plant RNA polymerases, the failure to secure specific transcription in vitro casts doubt on the proposition that all requisite proteins are known.

The known specific interaction of amatoxins with RNA polymerase II (Chochet-Meilhac and Chambon, 1974) provides

an advantageous alternative to harsh methods of protein purification. The extremely high affinity ($K_D=10^{-8}-10^{-10}$ M) and selectivity (RNA polymerase II vs III) of these peptides suggests their use in an affinity system for the isolation of transcriptional complexes; the chemistries and structure/activity relationships (Wieland, 1983) known for these compounds suggest several straightfoward approaches for their manipulation. Recently, for example, α-amanitin has been covalently linked to Sepharose 4B (Lutter and Faulstich, 1984) and shown to retain wheat germ RNA polymerase II. However, to desorb the enzyme, SDS elution was required (which also yielded proteins nonspecifically bound to the Sepharose), precluding subsequent in vitro assays. Further, the low yields in synthesis of the precious AMA-spacer and the exotic chemistries required to produce the matrix places a barrier between this approach and most researchers.

A system of greater flexibility than an insoluble amanitin is that of a soluble derivative linked to a useful biological ligand. Such a soluble ligand would have the advantages of being potentially higher in synthetic yield that the insoluble ligand. Additionally, the release of polymerase by inactivation of the amanitin attached to the soluble ligand using light of 314 nm (Lutter, 1982) could be more efficient than with an insoluble ligand, due to diffraction problems. But perhaps the greatest advantage is that of being able to modulate the concentration in vitro

and selectively immobilize not only class II RNA polymerases, but class III as well. An added and obvious benefit is the isolation of all amanitin-binding proteins present in wild type and mutant cells, including AMA transport proteins and the postulated amanitin-degrading oxidases.

To these ends, two approaches were used and are described below. One approach is to conjugate AMA via a spacer to Concanavalin A and exploit the affinity of this glycoprotein for glucosyl moieties in Sephadex G-75 or G-100 resins; this affinity may be easily disrupted by the addition of soluble glucose (Liener, 1976) and the AMA-coupled ConA is known to inhibit RNA polymerase II (Hencin, 1979). A second consideration is to link AMA via a spacer to iminobiotin and exploit the pH-dissociable interaction of this biotin derivative with avidin-agarose to retain the transcriptional complexes (Orr, 1981). The latter approach seemed especially feasible since Orr (1981) has retrieved iminobiotin-tagged erythrocyte membrane proteins on avidin-agarose from a population of other extraneous proteins.

Materials and Methods

Reagents and Amatoxins

Solvents used for syntheses and purification were hplc or analytical grade. Water used for all solutions was deionized and glass-distilled. α-Amanitin was a preparation of our laboratory and was purified as described (Little and

Preston, 1984). Azo derivatives of α-amanitin (ABGG and demeABGG respectively) were prepared by J.F. Preston according to the method of Preston et al. (1981) for ABGG (amanitinyl-azobenzoylglycylglycine); Demethyl ABGG (demeABGG or ^{3}H-ABGG) was derived from ABGG using $NaIO_4$ oxidation followed by $NaBH_4$ reduction of the afforded aldo derivative. To synthesize ^{3}H-labeled demeABGG, $NaB^{3}H_4$ was used for reduction according to a protocol formulated by J.F. Preston (manuscript in preparation). Amanitinyl-azobenzoyl-1-(aminohexanoyl-6-t-butyloxycarboxamide) or ADH-BOC was synthesized as described (Hencin and Preston, 1978).

Reagents for the synthesis of azo spacers and ligands were from Chemical Dynamics, Inc. (mono N-t-Boc-1, 6-diaminohexane hydrochloride, HDABOC; and N-t-BOC-Glycine-N-hydroxysuccinimide, BOCGLYNHS), Sigma Chemical Co. (2-Iminobiotin-N-hydroxy-succinimide hydrobromide, 2-IbNHS; Concanavalin A, Con A; avidin and avidin-agarose; [3-(Dimethylamino)-propyl]-ethyl carbodiimide HCL, EDC), Fisher Chemical Co. (Trifluoroacetic acid, TFA; Triethylamine, TEA) and Aldrich Chemical Co. (N,N'-dicyclohexylcarbodiimide, DCC). TEA (reagent grade) was vacuum redistilled from ninhydrin and stored in the dark at 4° C.

Chromatography and Solvents

Thin layer chromatography (tlc) was carried out on Silica Gel 60 F254 250 μ glass plates (EM Laboratories) or

on Whatman C18 reverse phase (250 μ) glass plates. Solvents for chromatography were $CHCl_3$: MeOH: H_2O (65:25:4) for normal phase or acetonitrile/0.5 M NaCl in varied ratios for reverse phase separations.

Spectroscopy

Ultraviolet absorbance spectra were recorded with a Beckman 25 recording spectrophotometer. Electron impact ionization mass spectra (EIMS) were obtained courtesy of Dr. Lester Taylor, Dept. Chemistry, Indiana University, Bloomington, IN.

Inhibition of RNA Polymerase II

Assays to measure K_I values of conjugates toward wheat germ RNA ploymerase II were as described in Section III using 1 unit of phosphocellulose-purified enzyme.

Preparation and Purification of ^{3}H-ABGGConA

ConA (Grade IV) which was purified by affinity chromatography on Sephadex G-100 was exhaustively dialyzed against distilled water and then lyophilized as described (Agrawal and Goldstein, 1965). ConA was redissolved in water and, after 1 h at room temperature, clarified by centrifugation at 12,000 x g for 30 min, and filtered through a 0.2 μ Millipore filter. Estimations of concentration for ConA utilized $E^{1\%}$=13.7 at 280 nm and an assumed protomeric molecular weight of 27,000 (Liener, 1976).

The covalent linkage of ^{3}H-ABGG to ConA was carried out by the method of Hencin, 1979 by J.F. Preston. ConA (0.02 μmole), ABGG(1.98 μmole) and ^{3}H-ABGG (2.9×10^{9} dpm/μmole, 0.02 μmole) were incubated in 1.8 ml 0.01 M sodium phosphate buffer (pH 5). After the addition of 100 μmole EDC as 0.2 ml, the reaction was allowed to proceed at room temperature for 6.3 h. The reaction mixture was then loaded onto a 0.9 cm x 46 cm column of Sephadex G-75 equilibrated in PBS^{+} (0.15 M phosphate buffered saline pH 7.5, 0.1 mM each $MnCl_2$ and $CaCl_2$) with the separation of unreacted material from the conjugate effected by chromatography in the same solvent. Affinity desorption of the conjugate (using 0.1 M glucose in PBS^{+}) was monitored by absorbance at 280 and 395 nm and by radioactivity. Following extensive dialysis versus PBS^{+} the peak fractions were lyophilized and then concentrated by resuspending in PBS+. Material used for the immobilization experiments had 1.6-1.7 ABGG molecules per ConA molecule, as measured by absorbance at 395 nm (E=14,000) for ABGG.

Synthesis, Purification and Characterization of PABGHDABOC

The synthesis of p-aminobenzoylglycyl-l-(aminohexanoyl-6-t-butyloxycarboxamide) or PABGHDABOC utilized the following protocol. To a stirring mixture of 1 mmole each of PABG (p-aminobenzoylglycine, U.S. Biochemicals) and HDABOC in 10 ml DMF (N,N'-dimethylformamide), two equivalents of TEA were added. The reaction was allowed to proceed for 48 h at

room temperature following the addition of 1 mmole of DCC. Dicyclohexylurea was filtered off, the filtrate taken to dryness *in vacuo*, and the mixture was redissolved in 10 ml methanol. PABGHDABOC was resolved from the majority of unreacted material by chromatography on Sephadex LH20 (4.2 cm x 56 cm) using methanol, with its elution position being 0.53-0.59 column volumes. Pure product (30% yield) was obtained by recrystallization from 50% aqueous methanol. Characterization of PABGHDABOC by normal phase tlc utilized detection by F254 attenuation, fluram reaction (Imai *et al.*, 1971), and chlorination (Pataki, 1963; Mazur *et al.*, 1962). Ultraviolet absorbance spectra were recorded in methanol and methanolic HCl and EIMS by direct probe insertion.

Synthesis and Purification of Iminobiotinyl Amatoxins

The conjugation of ADH to 2-iminobiotin was carried out as follows. One micromole of ADH-BOC that was evaporated *in vacuo* onto the inside of a 4 ml glass vial was resuspended in 0.3 ml TFA for 3 minutes. Excess TFA was removed *in vacuo* at 45° C with three 1 ml portions of absolute ethanol, such that no odor of TFA could finally be detected. To the dried ADH, 0.2 ml of 5 mM 2IbNHS in methanol and 0.1 ml TEA (10 mM in methanol) were added and the solution stirred for 4 h. Facile separation of ADH-2Ib was accomplished on Sep Pak C18 (Waters Chromatography Inc.) cartridges equilibrated with 20% acetonitrile/80% 0.5 M NaCl. In this case, ADH-2Ib was retained on the cartridge while unreacted ADH was not.

Product that eluted with 50% acetonitrile/50% NaCl showed retention on avidin-agarose at pH 9.5 (infra vide), inhibition of RNA polymerase II, and homogeneity (by visible azo detection) on reverse phase tlc (20% and 50% acetonitrile solvents described above).

To increase the spacer distance separating the amanitin and iminobiotin ligands, an alternative derivative was prepared. One hundred micromoles of PABGHDABOC was converted to the free amine as above for ADHBOC. One hundred-micromoles BOCGLYNHS in 1 ml methanol followed by 200 µmoles of TEA were added, and the mixture was stirred for 8 h. Fractionation on Sephadex LH20 (methanol, 0.9x44 cm column) revealed a product (rf-0.7 by F254 on normal phase tlc) different from the parent amine (rf-0.2). This product (56% yield) was shown to be pure (as above for PABGHDABOC) and used for the subsequent coupling to 2-iminobiotin.

Ten micromoles of PABGHDAGLYBOC were treated with TFA as above to remove the BOC protecting group. The free amine was reacted in 0.4 ml methanol with one equivalent of 2IbNHS and two equivalents of TEA. Following overnight reaction with stirring, the reaction was shown by reverse phase tlc (20% acetonitrile 180% 0.5 M NaCl) to be at least 80% complete, with PABGHDAGLY-2Ib at rf=0.17.

The arylamino spacer linked to 2-iminobiotin was coupled to AMA by diazotization as follows (J.E. Mullersman, personal communication). Two micromoles of PABGHDAGLY-2Ib (estimated from 80% yield) were removed from the previous

crude mixture, taken to dryness, and resuspended in 40 µl water (5° C). Next, 4 µl ice cold 0.5 M $NaNO_2$ were added and diazonium formation allowed to proceed on ice for 20 min. One-half of this was rapidly mixed with 1.0 µmole AMA (in 50 µl of 0.5 M $NaHCO_3$, pH 8), at which point the characteristic red azo color formed. Yield estimated from ^{395}A was 30%, with unreacted AMA and spacer also present and recoverable.

The AMAazoABGHDAGLY-2Ib was separated from reactants by affinity chromatography on a 7.5 ml column of avidin-agarose (Sigma, 1 µmole biotin capacity) preequilibrated in loading buffer (0.05 M $(NH_4)_2CO_3$ pH 9.5, 0.5 M NaCl, 3 mM BME). After a 5 column volume wash of the same buffer, the conjugate was desorbed with elution buffer (0.1 M ammonium acetate, pH 4.0, 0.5 M NaCl) and collected in a tube containing 1 M Tris-HCl, pH 7.9. This affinity purification was repeated after the conjugate was separated from buffer salts on a Sep Pak cartridge (using water for rinsing and 20% acetonitrile/80% 0.5 M NaCl for conjugate elution). Product from the second round of affinity purification was freed from buffer salts as just described and stored in 0.5 M NaCl.

Immobilization of RNA Polymerase II with ^{3}H-ABGGConA

Wheat germ RNA polymerase II as purified through phosphocellulose (Section III) was incubated (25° C, 25 min) with affinity purified ^{3}H-ABGGConA in a final volume of

250 µl. The final concentration of each was 2 x 10^{-7} M and 2.5 x 10^{-6} M respectively. This mixture was allowed to slowly percolate through a 0.8 ml column of Sephadex G-75 equilibrated with PBS^+; fractions of 5 drops (0.2 ml) were collected in microtiter plates. After several column volume washes with PBS^+, retained complexes were eluted with 0.1 M glucose in PBS^+. Fractions were assayed by counting 50 µl in Brays solution (Bray, 1960) and those of peak radioactivity were analyzed by polyacrylamide gel electrophoresis (Section III).

Immobilization of RNA Polymerase II with Iminobiotinylated Azoamatoxins

Wheat germ RNA polymerase II (0.3 nmoles, purified through the DE52 step according to Jendrisak, 1981) was incubated in 0.3 ml TEDG buffer with 4 nanomoles of AMAazoABGHDAGLY-2-Ib for 25 min at 25° C. Two volumes of sample preparation buffer (0.5 M $(NH_4)_2CO_3$, 0.5 M NaCl, 5 nM 2-mercaptoethanol) were added and the sample was placed on ice. The mixture was slowly percolated through a 0.5 ml column of avidin-agarose (at 4° C) equilibrated with loading buffer (supra vide) and the flow stopped to allow maximal binding. Fractions of 1.3 ml were collected as the column was washed with 5 volumes of loading buffer; subsequent elution of retained complexes was achieved by rinsing the column with elution buffer (supra vide) and collecting fractions as above. The absorbances of fractions were determined manually at 280 nm; flow-through and desorbed

peak fractions were dialyzed extensively against water and lyophilized. Lyophilized fractions were resuspended in 200 ul SDS denaturing buffer and analyzed by polyacrylamide gel electrophoresis.

Avidin Release from Inhibition of RNA Polymerase II

The in vitro assay of soluble avidin with RNA polymerase II and iminobiotinylated azoamatoxins at pH 9.5 was performed according to the following protocol. To a 12 x 75 mm test tube containing 50 µl pH 9.5 incubation buffer (5% glycerol, 100 µg/ml bovine serum albumin, 0.05 M $(NH_4)_2SO_4$, 0.05 M Tris-HCl, 3 mM BME, pH 9.5 at room temperature) was added 10 µl wheat germ RNA polymerase II (diluted 0.2 in pH 9.5 incubation buffer), 10-40 µl avidin (2 mg in 0.1 ml pH 9.5 incubation buffer) and 10 µl water or iminobiotinylated amatoxin. After an incubation of 25 min at 25° C, 10 µl of heat denatured calf thymus DNA (1.6 mg/ml) and 10 µl transcription mix $^{9.5}$ (contains all components used for assay at pH 7.9 as described in Section III, but prepared in water and lacking ammonium sulfate and Tris-HCl) were added and transcription was allowed to proceed for 4 min at 25° C. This reaction was terminated and TCA-insoluble counts determined as described in Section III.

Assays at pH 7.2 were performed as above except that transcription mixes were as in Section III. Buffers were 0.05 M Tris-HCl pH 7.2 at 25° C (pH 7.9 at 5° C).

Results

Azoamatoxins as Affinity Ligands: ABGGConA

Figure VI-1 presents a Dixon plot (see Section III) for inhibition of purified wheat germ RNA polymerase II by ABGG and demeABGG. The estimated K_I values are graphically estimated to be 3.5 nM and 4.5 nM respectively, indicating that 1) substituting into the 7' carbon of the aromatic ring of AMA does not detectably alter the interaction with plant RNA polymerases II and 2) no dramatic difference in affinity is evident by oxidation/reduction to provide demeABGG. Thus, the labeling of ConA with ^{3}H-ABGG should not diminish the inhibitory property relative to the parent ABGG coupled to such, and further that other azo derivatives (such as those of 2-iminobiotin) should be good candidates for affinity systems. Although not shown in Figure VI-1, the K_I of the ^{3}HABGGConA toward wheat germ RNA polymerase II was 18 nM. Clearly, while the affinity is altered fivefold, the conjugated ABGG is still a potent inhibitor and was next applied as an affinity ligand.

Figure VI-2A presents a typical profile of elution of the RNA polymerase II-^{3}H-ABGGConA complex from Sephadex G-75. As can be seen, radioactivity is associated with the flow through fractions (fractions 2-10) and those specifically eluted with 0.1 M glucose. However, the gel in Figure VI-2B shows that essentially no RNA polymerase II peptides (lane 7) are associated with either of the two main

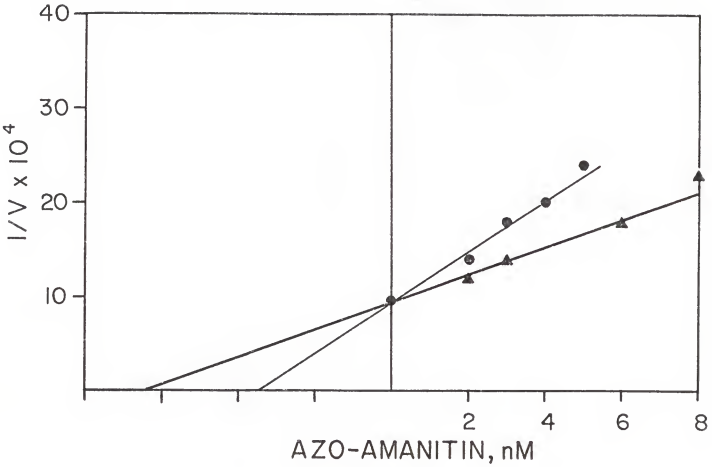
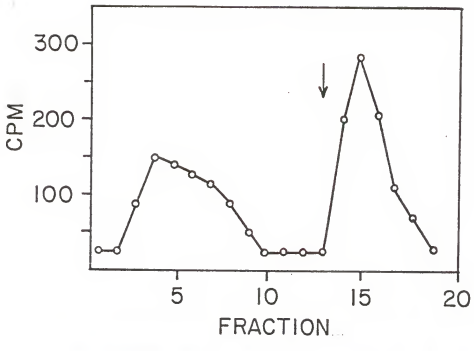


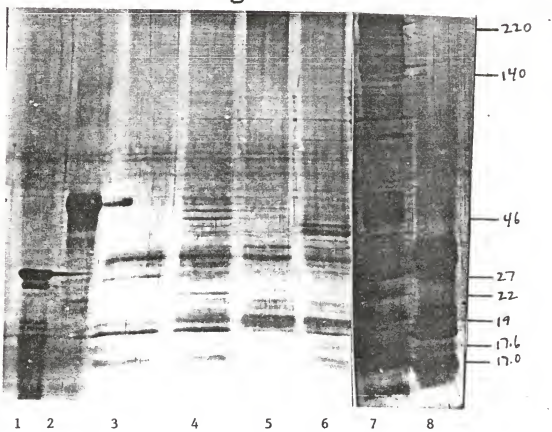
Figure VI-1. Azoamatoxin Inhibition of Plant RNA Polymerase II. Wheat germ RNA polymerase II was incubated with and assayed in the presence of either ABGG (circles) or demeABGG (trianges). Dixon plots show respective K_I values of 3.5 nM and 4.5 nM.

Figure VI-2. Immobilization of RNA Polymerase II on Sephadex G-75 Using ^{3}HABGG ConA. Figure VI-2a describes the elution from Sephadex G-75 of a ^{3}HABGG ConA-RNA polymerase II complex. The arrow indicates specific desorption with 0.1 M glucose. Figure VI-2b is a silver stained 5-15% polyacrylamide gradient gel of fractions from the G-75 Sephadex column. Entries on the gel are: Lanes 1 and 2, carbonic anhydrase (29 KD) and ovalbumin (45 Kd); lanes 3, 4, 5, 6 are Sephadex G-75 fractions 14, 15, 4, 5 respectively; lanes 7 and 8 are partially purified wheat germ RNA polymerase II and ^{3}HABGGConA respectively.

A


300
200
100
CPM
5
10
15
20
FRACTION

B


220
140
46
27
22
19
17.6
17.0
1
2
3
4
5
6
7
8

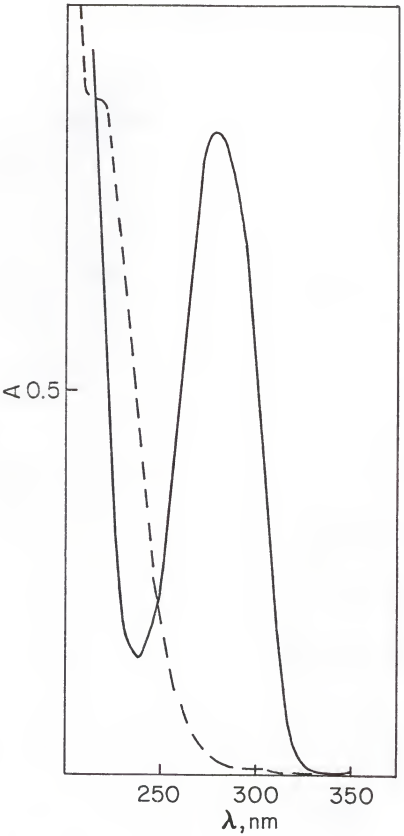
peaks. The other fractions in Figure VI-2A were not searched for RNA polymerase II peptides.

Synthesis and Characterization of PABGHDABOC and PABGHDAGLYBOC Spacers

Figure VI-3 presents the ultraviolet spectra of recrystallized PABGHDABOC in methanol and methanolic HCl. It is obvious that the carbodiimide condensation does not yield a derivative lacking a free arylamino group, which is needed for later diazotization into AMA. This fact is indicated by the shift of the absorbance spectrum to lower wavelengths in the presence of acid (forming the anilinium cation). The nature of the covalent linkage and the orientation of the condensed reactants is unequivocally shown in the mass spectrum of Figure VI-4. In addition to the expected molecular ion (392 m/z), sequence ions of m/z of 336, 319, 292, and 177 prove the primary structure of this peptide.

Figure VI-5 presents a composite thin layer chromatogram of the intermediates in the synthesis of PABGHDAGLYBOC. Lanes 1, 2, and 3 represent PABG, HDABOC, and PABGHDABOC respectively. Lane 4 is the free amine generated by removal of the BOC protecting group from PABGHDABOC; PABGHDAGLYBOC is shown in lane 5. All intermediates and products have thus acquired or gained the appropriate polar or nonpolar characters expected, as suggested by their relative mobilities in this solvent system. Since the PABGHDAGLYBOC showed the ultraviolet spectrum of Figure VI-3 and migrated the expected distance in Figure VI-5, it was not characterized

Figure VI-3. Ultraviolet Spectra of PABGHDABOC. Ultraviolet absorbance spectra of PABGHDABOC were recorded in 1.0 ml methanol with (dashed line) or without (solid line) the addition of 0.01 ml 12 M HCl.


A 0.5
250
300
350
λ, nm

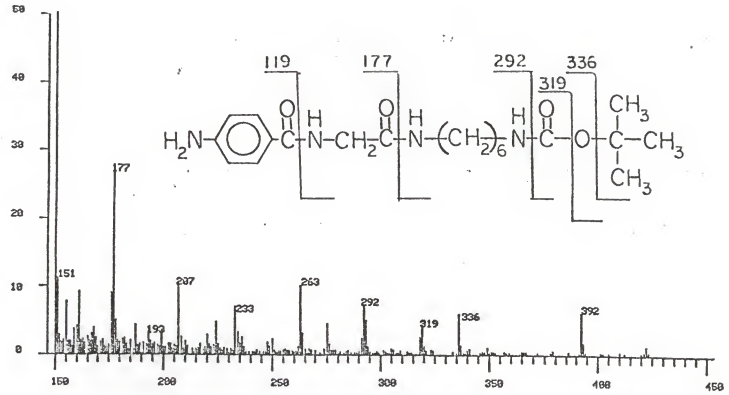


Figure VI-4. Mass Spectral Characterization of PABGHDABOC.
The electron impact mass spectrum of PABGHDABOC is shown. The molecular ion (M/Z 392) along with the indicated sequence ions prove the nature of conjugation between PABG and HDABOC.

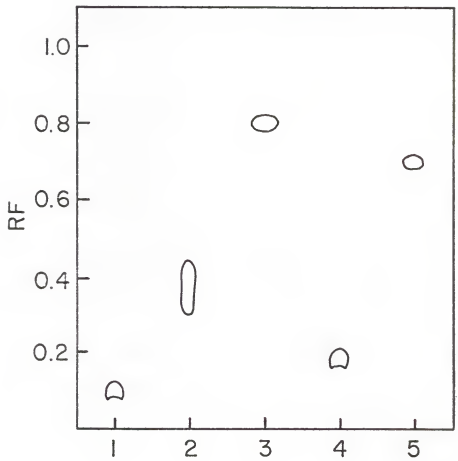


Figure VI-5. Mobilities of PABG Derivatives on TLC. Relative mobilities on normal phase tlc are shown for PABG and derivatives. Solutions of 5 µl spotted in positions 1-5 are respectively: PABG, HDABOC, PABGHDABOC, PABGHDA, and PABGHDAGLYBOC. The detection of PABG derivatives was by F254 and HDABOC was visualized by chlorination.

further, but was derivatized to provide the iminobiotinylated spacer shown in Figure VI-6.

Iminobiotinylated Amatoxins as Affinity Ligands

The demonstration that ADH-2Ib is an effective inhibitor at pH 7.2 and pH 9.5 is given in Table VI-1. In these studies, the level of inhibition of RNA polymerase II mediated by ADH-2Ib at pH 7.2 is not overcome by the addition of soluble avidin (reactions 1-2); however the addition of soluble avidin at pH 9.5 (reactions 4-7) abolishes the inhibition by this ligand. This experiment thus shows that, while the ADH-2Ib conjugate can inhibit RNA polymerase II at the pH (9.5) needed for immobilization on avidin-agarose, the ligand spacer may be of insufficient length. This table also demonstrates that RNA polymerase II is capable of tolerating the higher pH needed for immobilization and that this activity is stimulated approximately 40% over that at pH 7.2.

A comparison of the efficiency of the iminobiotinylated amatoxins, ADH-2Ib and AMA-ABGHDAGLY-2Ib, at pH 9.5 is given in Table VI-2. In both cases, despite the difference in length of spacers (7 atoms) used to make these derivatives (see Figure VI-7), soluble avidin at pH 9.5 restores RNA polymerase II activity to control levels or better. Inhibition (K_I) values for each azo derivative were 20 nM.

Figure VI-8A describes the elution of partially purified RNA polymerase II and AMA-ABGHDAGLY-2Ib preformed

Figure VI-6. Synthetic Scheme for Iminobiotinylated Spacers.
The construction of diazotizable spacers begins with the condensation of PABG and HDABOC. Subsequent coupling of additional glycines can increase the length of the spacer, before the addition of 2-iminobiotin.

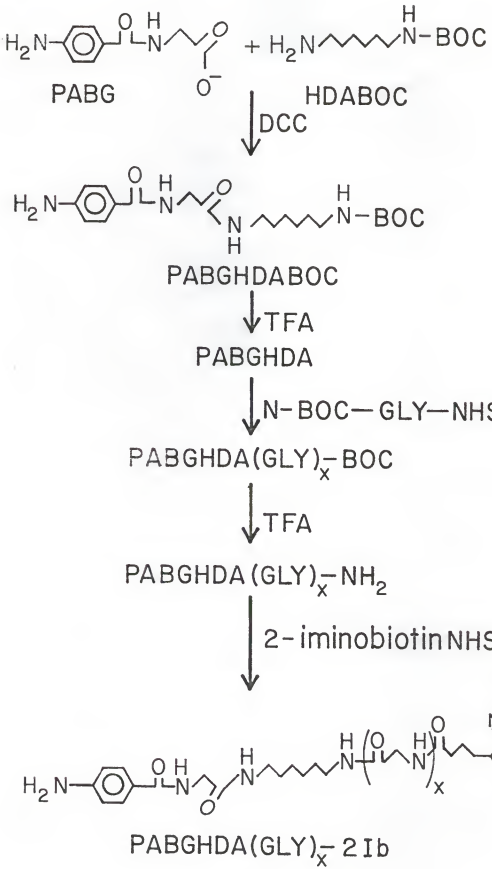
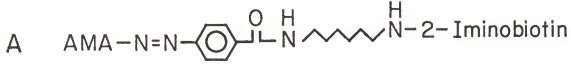

PABG
HDABOC
DCC
PABGHDABOC
TFA
PABGHDA
N-BOC-GLY-NHS
PABGHDA(GLY)x-BOC
TFA
PABGHDA(GLY)x-NH2
2-iminobiotin NHS
PABGHDA(GLY)x-2Ib

Table VI-1. Effect of pH on the Interaction of RNA Polymerase II with ADH-2-Ib.

Reaction	pH	ADH-2-Ib	Avidin	Cpm	% Control
1	7.2	-	-	3469	100
2	"	+	-	1056	30
3	"	+	+	866	25
4	9.5	-	-	4935	100
5	"	-	+	6443	130
6	"	+	-	1008	20
7	"	+	+	4370	89

Table VI-2. Release from Amatoxin Inhibition of RNA Polymerase II by Soluble Avidin at pH 9.5.

Reaction	ADH-2-Ib	AMA-ABGHDAGLY-2-Ib	Avidin	Cpm	% Control
1	-	-	-	7995	100
2	+	-	-	5085	64
3	+	-	10µl	9997	125
4	-	+	-	3755	47
5	-	+	10µl	7811	98
6	-	+	20µl	8950	112
7	-	+	40µl	8698	109



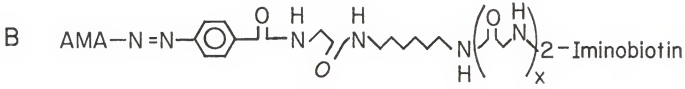
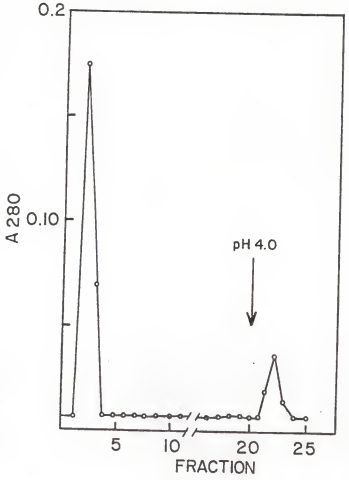
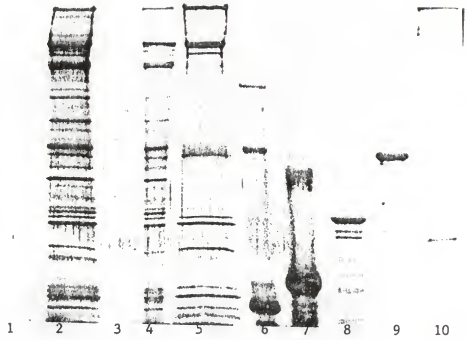


Figure VI-7. Structures of Iminobiotinylated Amatoxins.
A comparison of the spacer lengths of ADH-α-Ib (Figure 7a) and AMA-ABGHDAGLY-α-Ib (Figure 7b) is given.

Figure VI-8. Immobilization of RNA Polymerase II with AMA-ABGHDAGLY-2-Ib.
RNA polymerase II incubated with AMA-ABGHDAGLY-2-Ib was percolated through a column of avidin-agarose and the column was washed with loading buffer. At fraction 20, specific elution at pH 4.0 was effected (Figure 8a). Fractions from the column were analyzed by polyacrylamide gel electrophoresis (5-20% gradient gel) in Figure 8b. Entries in the various lanes are: lanes 1 and 3: retained fraction, 0.05 ml and 0.01 ml respectively; lanes 2 and 4: flow through 0.05 ml and 0.01 ml respectively; lane 5: wheat germ RNA polymerase II; lane 6: lysozyme (14.3 kd); lane 7: lactoglobulin (18.4 kd); lane 8: carbonic anhydrase (29 kd); lane 9: actin (46 kd); lane 10: myosin (220 kd) and phosphorylase B (97.4 kd).


0.2
0.10
A 280
pH 4.0
5
10
20
25
FRACTION


1
2
3
4
5
6
7
8
9
10

complexes from avidin-agarose. As can be seen in the gel of Figure VI-8B, although a few faint bands corresponding to RNA polymerase II peptides are seen to be retained (lane 1) the majority are present in the flow through fractions. Thus, the in vitro activity toward avidin seen for this conjugate is reflected using insoluble avidin.

Discussion

The incentive to consider ^{3}HABGGConA as a tractable ligand system was based on the ease with which the ligand could be bound to Sephadex matrices and subsequently desorbed. Further, the ability of this conjugate to kill cells (Hencin, 1978)--via receptor-mediated uptake--through presumed interaction with RNA polymerase II, as well as the inhibition K_I (18 nM) seen with this conjugate would suggest that the AMA moiety can interact with RNA polymerase II, despite its linkage to a macromolecule (100,000 daltons, Liener, 1976). Thus, the covalent linkage of ABGG to ConA (which results in at least 70% amide bonds; (Hencin and Preston, unpublished) and subsequent affinity purification should provide a stable conjugate retaining both inhibitory activity toward RNA polymerase II and Sephadex binding. Further, since the gel in Figure VI-2 (lane 8) shows no crosslinking of the ConA under the conditions used to couple ABGG, disruption of the RNA polymerase II-ABGGConA-Sephadex interaction by steric hindrance would not necessarily be expected.

The gel in Figure VI-2 demonstrates, in fact, that immobilization of RNA polymerase II-ABGGConA on Sephadex may be a difficult system to use. The expected convenience of the radioactive label to monitor elution of the enzyme is not achieved, since RNA polymerase II peptides were not found associated with either fraction. An additional problem with this system is that ^{3}HABGGConA, although affinity-purified on the same matrix, consistently yielded both a flow through fraction and a desorbed fraction (not shown). This was the case even in the absence of RNA polymerase II and appeared when ^{3}HABGGConA was incubated with RNA polymerase II buffer (TEDG) only. Since addition of Ca^{2+} and Mn^{2+} or deletion of EDTA from this buffer did not restore quantitative and specific elution, and because RNA polymerase II is chromatographically unstable without the other buffer components, the ligand system employing ABGG coupled to ConA was not further pursued.

Since the K_I information given by Figure VI-1 shows that the acquisition of greater than 9 atoms on the indole ring of AMA does not abolish interaction with RNA polymerase II, a logical deduction was to consider the synthesis of a spacer that might link AMA with alternative ligands, such as iminobiotin. However, since both ABGG and iminobiotin possess only reactive carboxyl groups and since ABGG would not necessarily be readily available to other researchers, the synthesis of a diazotizable spacer molecule (containing iminobiotin) that could be undertaken with

commercially available materials by almost anyone was begun. Hence the coupling of PABG to HDABOC, NHSGLYBOC, and eventually, 2-IbNHS.

Figures VI-3 and VI-4 show that PABG and HDABOC may be readily coupled in organic solvents with DCC to provide a diazotizable spacer with a blocking group that is easily removed for further extension of the chain. Both figures show that although the amino group of the HDABOC may condense with carboxyl groups, the participation of the arylamino nitrogen lone electron pair in the aromatic system, precludes its nucleophilicity. Additionally, the BOC group on PABGHDABOC may be stable during diazotization (not shown), such that coupling of this to AMA can precede the attachment of ligands.

One curious aspect of this synthesis was that the yields of several preparations whether in DMF or methanol never exceeded 30%. Unreacted HDABOC, PABG, and carboxyl-activated PABG could be found by tlc or Sephadex LH20 chromatography, even when all reactants were initially present at 0.1 M concentration. Extending the reaction time up to one week or attempting to drive the reaction, by frequent filtration of insoluble dicyclohexylurea or addition of excess TEA, did not alter the yields. Presently, no explanation seems suitable, except for perhaps an aggregation of reactants into a physical state that prevents complete coupling. However, the ease of scale-up and recovery of reactants make this an economical reaction.

Figure VI-6 describes the general synthesis of PABGHDABOC and PABGHDABOC-based linkers to couple AMA to iminobiotin. The NHS esters of glycine and iminobiotin provide a facile and uncomplicated route to the synthesis of the final product and one where carbodiimide coupling is therefore unnecessary. This is important for two reasons. One is that iminobiotin has a basic guanidino group that could be coupled through the biotin ring, yielding a derivative that would likely be useless (Heney and Orr, 1981), were carbodiimide to be used. A second reason is that attempts to couple N-t-BOC-heptaglycine to PABGHDA by carbodiimide in a single step were unsuccessful. The acidity of the hydrogens and absence of bulky substituents on the α-carbon of glycine may have, in this case, allowed numerous reactions to occur, none lending a defined species. The commercial availability of the above esters, ease of monitoring the adducts coupled to PABG by F254 attenuation (tlc), and defined species (see Figure VI-5) produced through this coupling are a decided advantage of this approach. A more redeeming feature is that homologous linkers, each differing by a single glycine, can be constructed and distances representing the approximate Stokes radius of the amanitin binding site can be estimated (infra vide).

The tractability and limits of the iminobiotinylated amatoxins as potential ligands are illustrated by Tables VI-1 and VI-2 and also Figure VI-8. It is clear that both

ADH-2Ib and AMA-ABGHDAGLY-2Ib are good inhibitors of RNA polymerase II (K_I=20 nM, not shown) even though neither can immobilize the enzyme on avidin-agarose. Given the *in vitro* data at pH 9.5 the clearest explanation for this is that the iminobiotin-avidin affinity ($K_D=10^{-15}$ M; Heney and Orr, 1981), being orders of magnitude greater than that of RNA polymerase II for α-amanitin ($K_D=10^{-8}$ to 10^{-10} M), is able to exploit the steric crowding that apparently exists between the avidin (67,000 daltons, Bayer and Wilchek, 1980) and RNA polymerase II (500,000 daltons; Guilfoyle, 1981) and secure the amanitin away from the polymerase.

To understand the extent of this crowding, a comparison of the lengths of spacers on these two iminobiotinylated amanitins is shown in Figure VI-7. Although a primary sequence difference exists between the two, the most obvious difference is the 6 atoms, provided by two glycines that are lacking in the ADH-2-Ib. Green *et al.* (1971) showed that bifunctional biotin molecules separated by varied numbers of methylenes could form dimers with avidin if 16 or more atoms separated the two carboxyl groups of the biotins; importantly, this implies that at least 8 atoms are required per avidin in order to get stable dimers, and perhaps defines the contact radius of avidin. The conjugation of AMA to PABG yielding AMA-ABG provides an azo derivative (K_I=2.4 nM for calf thymus RNA polymerase II) that has been coupled to several IgG carriers; the lowest K_I of one of these is 4.6 nM, indicating that the contact radius of RNA polymerase

II with a molecule (IgG, 150 Kilodaltons) only slightly larger than avidin may minimally be overcome by a spacer the length of PABG. Considering these collective spatial requirements of RNA polymerase II and avidin for interaction with their respective ligands, it is not surprising to see ADH-2-Ib fail as an affinity ligand. An examination of the structure of AMAABGHDAGLY-2-Ib shows that 3 atoms (less PABG and 8 atoms required by avidin) separate the these two contacts on the spacer. Although this distance should be adequate, immobilization does not occur. A spacer with two additional glycines (total length 3.64 nm) did not successfully immobilize RNA polymerase II and avidin demonstrated *in vitro* release of inhibition as with the other derivatives (data not shown). Since 0.8 nm of this spacer would be buried within the avidin, one can conclude that the domains of RNA polymerase II which interact with the indole ring on AMA may be hidden from the protein surface by more than 2.8 nm.

An important observation from these experiments provides a basis for using iminobiotinylated-substituted amatoxins as retrievable ligands. Published pH optima for RNA polymerases appear to be pH 8.0, with less than 10% activity at pH 9.5 (Polya and Jagendorf, 1971). This initially suggested that either the enzyme would not tolerate the higher pH or that assays at pH 9.5 would be difficult. I observed while formulating the transcription mixture for pH 9.5 that Mn^{2+} rapidly formed a dark brown

precipitate (probably the oxyhydroxide) indicative of Mn^{3+}; this was not the case if Mn^{2+} was first mixed with XTP's to form the chelated complex, and this mixture even stimulated transcription at the higher pH. Thus it appears that the pH profiles indicating a lower optima may instead profile the pH at which Mn^{2+} is not oxidized, rather than that of true enzyme activity. Collectively, this implies the need for caution in interpreting such experiments and, at the same time, demonstrates that RNA polymerase II will tolerate such pH extremes. Thus substitutions of iminobiotin into other positions of the amatoxin molecule may lend a true retrievable ligand for RNA polymerase II.

SECTION VII

CONCLUSIONS

These studies have indicated the ready potential of isolating RNA polymerase II mutants in plants. Background problems associated with inhibitor degradation may be eliminated with the choice of an appropriate amatoxin. Carrot cell lines resistant to meAMA were obtained that lacked degradative capacity toward the selecting agent. The improved synthetic yields of meAMA should now permit an extension of this approach to other plant systems.

The selective retrieval of RNA polymerase II using AMA coupled to soluble entities has addressed the feasibility of this approach and identified potential problems. The inability to retrieve RNA polymerase II using the spacer lengths described suggests an approach using substitutions into the amatoxin other than diazotization. The fact that RNA polymerase II tolerates the basic conditions required for immobilization identifies iminobiotin-based ligands as having potential for the selective retrieval of RNA polymerase II if appended to other residues in the amatoxin molecule.

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